Diagnostic and therapeutic biomarker responses in HIV and tuberculosis co-infected patients

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c. Mthiyane T, Pym A, Dheda K, Rustomjee R, Reddy T, Manie S. Longitudinal assessment of health related quality of life of HIV infected patients treated for tuberculosis and HIV in a high burden setting. Quality of Life Research. 2016 Dec 1;25(12):3067-76.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
ATT	Anti-TB treatment
AUC	Area under the concentration curve
BCG	Bacille Calmette-Guérin
CD	Cluster of differentiation
CFP	Culture filtration protein
CNS	Central nervous system
COA	Clinical outcome assessment
COOP	Cooperative Information Project
CYP	Cytochrome P450
DME	Drug metabolising enzyme
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
EQ	Euro-Quality of life
ESAT-6	Early secreted antigenic target protein 6
FACT	Functional Assessment of Cancer Therapy
FAHI	Functional Assessment of HIV Infection
GHSA	General Health Self-Assessment
GIT	Gold in tube

HAART	Highly Active Antiretroviral Therapy
HAQ	AIDS Health Assessment Questionnaire
HAT	HIV/AIDS Quality of Life
HIV	Human immunodeficiency virus
HOPES	HIV Overview Problems Evaluation System
HRQOL	Health-related quality-of-life
HUI	Health Utility Index
ICU	Intensive care unit
IFN-γ	Interferon gamma
IGRA	Interferon-gamma release assay
IL	Interleukin
INH	Isoniazid
IQR	Interquartile range
IU	International units
IUATLD	International Union against TB and Lung Disease
LAM	Lipoarabinomannan
LASA	Linear Analogue Self-Assessment
LF	Lateral flow
LTBI	Latent tuberculosis infection
MDR	Multi-drug resistant
MHIQ	McMaster Health Index Questionnaire
MID	Minimal important difference
MOS	Medical Outcomes Study Short Form-36
MQOL	Multidimensional Quality of Life Questionnaire for HIV/AIDS
MTB	Mycobacterium tuberculosis

MTSE	Murine tracheal surface epithelial
MW	Molecular weight
NAT	N-acetyl transferase 2
NATP	N-acetyl transferase pseudogene
NHP	Nottingham Health Profile
NIH	National Institute of Health
NNRTI	Non-nucleoside reverse transcriptase inhibitors
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI	Protease inhibitors
PK	Pharmacokinetic
PPD	Purified protein derivative
PTB	Pulmonary tuberculosis
PZA	Pyrazinamide
QFT	Quantiferon
QOL	Quality of life
QWB	Quality of Wellbeing
RIF	Rifampicin
RNLI	Normal Living Index
SAE	Serious adverse event
SAPIT	The Starting Antiretroviral Therapy at Three Points in Tuberculosis
SF	Short Form
SIP	Sickness impact profile
SNP	Single nucleotide polymorphism
TB	Tuberculosis

TPT	Tuberculosis preventive therapy
TST	Tuberculin skin test
VAS	Visual analogue scale
WHO	World Health Organization
WHOQOL	World Health Organization Quality of Life

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Abstract

Introduction: Biomarkers of tuberculosis (TB) diagnosis and treatment response in patients co-infected with human immunodeficiency virus (HIV) are a necessity to ensure early diagnosis and adequate monitoring of TB treatment response. We conducted 3 sub-studies: study 1 was a bioavailability study; study 2 was a PK study in HIV-TB co-infected persons, and study 3 evaluated a WHO-recommended treatment algorithm in TB-HIV co-infected persons. Study 1 and 2 contributed to the study of 2 (*NAT2*) polymorphisms. Study 1 was leveraged to evaluate Quantiferon Gold in tube (QFT-GIT) and a quality of life instrument as a longitudinal biomarker in smear and culture positive TB-HIV co-infected patients. Study 3 was leveraged to study urine lipoarabinomannan (LAM) as a diagnostic adjunct in smear-negative HIV-infected patients treated for TB.

Methods: Blood was collected from participants with HIV-infection only and TB-HIV co-infection for *NAT2* polymorphisms at baseline, and for QFT-GIT at baseline, month 3, 6 and 12; a health-related quality-of-life (HRQOL) instrument was applied at the same timepoints to monitor treatment response in Study 1. An additional 40 TB-HIV co-infected participants (Study 2) were included in the analysis for the assessment of *NAT2* polymorphisms and its effect on isoniazid plasma levels and hepatotoxicity. Urine was collected from seriously ill HIV-infected patients with confirmed smear-negative presumptive-TB (Study 3) prior to anti-TB treatment and tested using a commercially available LAM-ELISA. Blood and sputum were collected and processed for TB culture.

Results: One hundred and twenty participants (100 TB-HIV co-infected and 20 non-TB but HIV-infected) from Study 1 and Study 2 with genotype results and were evaluated. Percentage of metabolisers in each category were: slow 52.5% (63/120), (*NAT2*5/*5*); intermediate 35.8% (43/120), (*NAT2*4/*5* and *NAT2*5/12*); and rapid 11.7% (14/120), (*NAT2*4/*11*, *NAT2*11/12* and *NAT2*12/12*). In general, isoniazid area under the concentration curve $(AUC)_{0-\infty}$ and maximum concentration (C_{max}) were lower amongst the study 1 compared to study 2 participants. INH and AcINH PK parameters across genotypes were not statistically significantly different within each study. The log AcINH: log INH ratio, calculated as a measure of acetylation at two and four hours post-dose, showed no statistically significant difference between genotypes.

Baseline, month-3, and month-6 interferon gamma (IFN-γ) responses, irrespective of antiretroviral therapy, did not differ between TB patients who culture-converted and those who did not (1.25 vs. 1.05 IU/ml, p=0.5; 3.76 vs. 1.15 IU/ml, p=0.2; 0.06 vs. 0.7, p=0.3 respectively). IFN-γ levels did not correlate

with markers of sputum bacillary load (p=0.50 and 0.30 for smear status and liquid culture time-to-positivity, respectively).

Baseline HRQOL scores were analysed in 76 participants. Those with CD4 count <200 had lower HRQOL when compared to those with CD4 count >200 [99.65 (29.68) versus 124.46 (29.02); p<0.001]. There was a significant improvement in total score between baseline, month 3, 6 and 12 (all p-values<0.0001) for all groups. There was a significant increase in the total score over time (Slope: 3.45 (2.77, 4.1) p-value <0.001).

Data from 156 patients (Study 3) with complete data were analysed for LAM. Mean age was 37 years, and median CD4-count was 75 cells/mm^3 (IQR [34–169]). 54/156 (34.6%) were sputum culture-positive, 12/54 (22%) blood-culture positive, and 53/156 (34.0%) LAM-positive. Thus, LAM sensitivity was 55.6% (30/54). The study design did not allow for calculation of specificity. Urine-LAM positivity was associated with low CD4 count (p=0.002). Ninety percent (48/53) of LAM-positive patients received antibiotics [15/48 (31.2%), 23/48 (47.9%) and 10/48 (20.8%) received one, two or three different antibiotics respectively], while the duration of antibiotic therapy was more than 5 days in 26 of 46 (56.9%) patients.

Conclusion: 1) Although there was a high prevalence of slow acetylation compared to results of other studies in South Africa, this did not translate to significantly higher rate of hepatic adverse events in this group. There was no direct concordance between genotype and phenotype. *NAT2* genotyping is therefore not a useful tool in individualised treatment allocation among this group. More work is needed to investigate NAT2 as a biomarker for hepatotoxicity in patients receiving isoniazid, and to explore polymorphisms and other factors better describing acetylator phenotype in this population.

2) QFT-GIT is not a useful biomarker for monitoring TB treatment as reversion does not always occur during and at the end of TB treatment.

3) HRQOL is a useful instrument for evaluating TB-HIV treatment response. Further studies are needed with more frequent evaluations and using more specific HRQOL instruments.

4)) Urine LAM is a useful biomarker for diagnosis of smear-negative TB in patients with low CD4 counts. Early diagnosis with urine LAM could potentially reduce excessive use of antimycobacterial antibiotics and thereby assist in antibiotic stewardship.

Diagnostic and therapeutic biomarker responses in HIV and tuberculosis co-infected patients.

Chapter 1

1. Introduction

Tuberculosis (TB) remains a leading cause of global morbidity and mortality, with approximately 10 million cases and 1.5 million deaths in 2018 (1). South Africa is a high TB burden country with an estimated 301,000 cases in 2018, and is counted among the eight countries accounting for two thirds of the global total of tuberculosis (TB) (1). In addition, South Africa is one of the fourteen countries with overlap in high burden of drug susceptible TB, Multi-drug resistant (MDR) TB, and TB/ human immunodeficiency virus (HIV). There is country commitment to work towards ending TB by 2035 (2), however this combination makes it difficult to achieve the targets to end TB by 2035 (3). KwaZulu Natal is one of the provinces in South Africa with the highest TB prevalence, including drug resistant TB (4).

To achieve the targets to end TB, early diagnosis of TB and proper monitoring of treatment to ensure cure are necessary. Diagnosis of TB in HIV-infected patients and monitoring of treatment is difficult. Researchers have found that HIV-infected individuals tend to have smear-negative TB, depending on the level of immunosuppression (5). Chest X-rays are not conclusive for diagnosis of TB, more so in HIV-infected patients, as chest X-rays are atypical. Biomarkers of TB-HIV diagnosis and treatment monitoring are needed to ensure early diagnosis and treatment, as well as adequate monitoring of treatment responses.

Besides early diagnosis and treatment, monitoring of patients on combined TB and HIV therapy is one of the most important aspects of management of these two co-morbidities. TB treatment on its own presents with multiple problems, as the drugs used for its cure have multiple side effects affecting different organs (6). In addition, HIV therapy has overlapping side effects with TB treatment (7), and a combination of these therapies require strict monitoring to ensure patient safety. Some patients default treatment because they cannot handle the side effects they experience with the treatment (8-10), whilst some patients find the treatment completely tolerable, without experiencing any side effects (11). In sputum smear-negative individuals with HIV infection, TB is even more difficult to diagnose. This has often led to giving these patients antimicrobial antibiotics while waiting for sputum culture results. This, in spite of the WHO recommended algorithm to reduce mortality in seriously-ill patients with HIV infection and smear-negative pulmonary TB, recommending the start of anti-TB treatment (ATT) within three days of

admission (12). When patients are given these treatment regimens there should be proper monitoring of treatment response and side effects. There are different biomarkers and tools of monitoring TB treatment responses and evaluating the side effects of TB and HIV treatment or predicting which patients are more likely to experience these side effects. These may include genetic tests, quality of life tools, other tests on different body fluids and self-reporting by patients.

In this Chapter the following points will be addressed, 1.1 Thesis rationale, 1.2 Problem statement, 1.3 Research question, 1.4 Aims and objectives, 1.5 Studies and methods reported in this thesis, 1.6 Definition of terms and 1.7 Justification and outline of the thesis.

1.1 Thesis rationale

TB is one of the commonest opportunistic infections in people living with HIV, and is known to be the leading cause of morbidity and mortality (13, 14). In South Africa, more than 70% of patients with TB are also HIV-infected (15), and about 50% of them receive antiretroviral therapy (ART) (1). Guidelines for TB and HIV treatment have been changed to allow all HIV-infected TB patients to be started on ART irrespective of their CD4 count, in line with the latest World Health Organization (WHO) and country guidelines. These guidelines require all TB patients to receive ART with their TB treatment, as ART reduces opportunistic infections as well as morbidity and mortality (16, 17). TB programmes in South Africa and elsewhere use defined clinical outcomes such as cure (smear-negative at treatment completion and on at least one previous occasion), plus treatment completion without confirmation by smear-microscopy, loss to follow up, treatment relapse and chest radiography to assess TB treatment effect.

Diagnosis of TB and monitoring of TB treatment response using sputum smear and culture may be ideal but poses many problems as HIV-infected patients are frequently smear-negative (18), or may fail to produce sputum (19), and these tests themselves are suboptimal predictors of outcome (20). Gene Xpert MTB/RIF, while useful for diagnosis of TB, is not as beneficial in patients previously treated for TB as it detects both live and dead bacilli (21, 22). Urine lipoarabinomannan (LAM) has also been investigated as a biomarker for the diagnosis of TB and has been found to work best in HIV-infected patients with severe immunosuppression (23, 24). We chose to investigate the usefulness of urine LAM and its benefits in diagnosis of seriously ill, smear-negative TB HIV-infected patients.

Failure to respond to treatment has serious consequences, such as infectiousness and increased morbidity and mortality (25). Genetic factors have a role to play in how individuals respond to treatment (26). Much has been done to assess the extent to which genetics and environmental factors affect patients' response to treatment. Genomic variation or single nucleotide polymorphisms (SNPs) are responsible for diversity in human subjects (27). Isoniazid (INH) is one of the backbones of the combination regimen for treatment of TB. The bacterial activity of INH has been studied extensively (28-32), and is highly efficient, especially during the first few days of TB treatment. The rate of acetylation of INH by *NAT2*, an important enzyme responsible for the metabolism of certain antibiotics, (28, 33-37) and inter-individual variation in plasma concentration has been studied extensively over the years. *NAT2* is one of the three *NAT* genes involved in drug metabolism and the most important for INH metabolism; the other two are *NAT1* and NAT pseudogene (*NATP)* (38). As reported by Parkin et al., there is a trimodal distribution of metabolic phenotype for isoniazid reported in different populations consisting of rapid, intermediate and slow acetylator status (39).

The consequences of pharmacogenetic variation in acetylation enzymes include (i) altered drug levels of INH; (ii) altered drug interactions due to changes in the concentration of the perpetrator drug (and NAT substrate); and (iii) adverse drug reactions (40). The speed of acetylation largely determines which individuals develop serious adverse effects of drugs (slow acetylators) or poor response to treatment (rapid acetylators). Of note are the INH induced hepatotoxicity and peripheral neuropathy experienced by slow acetylators that have been reported (41-51). Drug-induced hepatotoxicity is one of the main adverse events that has been reported to be a result of the individual's acetylator status (41, 52-55). The clinical significance of these variations leads to the prediction of exposure, with certain individuals being more prone to adverse events or certain diseases. The functional importance of *NAT2* genotype has been well-investigated in Caucasian and Asian populations (27, 41, 56-62), while information on black South Africans is scanty (63-66). We therefore decided to investigate the effects of NAT2 on the metabolism of INH in the Black, Zulu-speaking population.

Use of TB treatment monitoring tools that give results in a short time, allowing access of results within the same day, and that are more specific, is ideal. More recently interferon-gamma release assays (IGRAs) have become available and have been investigated for use as diagnostics and biomarkers of TB treatment response and prognosis (67-87). Reports that IGRAs may be useful in monitoring TB treatment response have been mentioned in some studies (84, 88). While in other studies, most patients remain IGRA-positive

after completion of ATT, despite sputum smear and culture conversion (73). These studies have examined the utility of measuring longitudinal interferon-gamma (IFN-γ) responses during TB treatment. They have mostly been conducted in pre-dominantly HIV-uninfected individuals (73, 82, 89-91) and mainly in countries with low TB and HIV burdens (72, 92-94). Only a few studies have used the commercially available standardised QuantiFERON®-TB Gold in Tube (QFT-GIT) assay in settings with high burden of TB and HIV (85, 95). However, to our knowledge, no study has hitherto reported effects of ART and or ATT on quantitative IFN-$_{\gamma}$ responses in TB-HIV co-infected patients. There are also hardly any data about the effect of ART alone on such responses. A further limitation of these studies is that several published studies, even from Africa, have used non-standardised in-house IGRAs in an enzyme-linked immunosorbent assay format (77, 96). Although there have been reports that IGRAs may correlate with TB bacterial load in low burden settings (97, 98), this was not the case in the only published report in patients from a high burden setting (85). Therefore, there was a need for more data from high burden settings, such as South Africa, where TB prevalence was estimated to be between 0.4 million and 0.6 million cases in 2012 (13).

These monitoring tools may satisfy the biomedical model but not the biopsychosocial model of TB treatment. A comprehensive assessment of the health status of patients is essential, including routine clinical, radiological, bacteriological and psychosocial assessments, in considering the impact TB has on health and patients' perception of their wellbeing (99). The possibility of side effects when receiving TB treatment (100, 101), may be worsened by concomitant HIV treatment, thereby further affecting patients' quality of life (QOL). Health-related quality of life (HRQOL) in patients receiving treatment for TB or those receiving treatment for HIV have been investigated in different studies (102-108). However, studies investigating the HRQOL of patients co-infected with TB and HIV and receiving concomitant therapy are few and far between (109-111).

Decision-making by clinicians whether to alter treatment may result from reported and or observed adverse effects of treatment or based on the extent to which the patient's QOL has been modified by the disease. HRQOL may be included, which has the potential to give a fuller picture of the patients' state of health, from the beginning to beyond the end of TB treatment. According to Dhuria et al., this can be performed by measuring the HRQOL that has several dimensions (112). HRQOL has been defined as how well people are capable of performing activities of daily living and their well-being (113).

The purpose of this thesis is to report on the studies conducted to assess biomarkers of TB diagnosis and responses to ATT alone, and combined ATT and ART. The aims of these sub-studies were 1) to study the relationship between polymorphisms in drug metabolising enzymes and drug pharmacokinetics and hepatotoxicity; 2) to investigate the use of an INF-γ release assay to determine the response to TB therapy and 3) to assess the HRQOL of TB and HIV on dual therapy. 4) to investigate the usefulness of urine LAM to diagnose TB in smear-negative seriously-ill patients with HIV-infection and investigated the use of mycobacterial antibiotics in these patients.

1.2 Problem statement

TB is one of the world's diseases with high morbidity and mortality. In 2016, 6.3 million new cases of TB were reported (an increase from 6.1 million in 2015), equivalent to 61% of the estimated incidence of 10.4 million, and 10.1 million in 2018 (1). In 2018, according to the WHO Global TB report, Africa had an estimated 24% of the global TB incidence, with South Africa having 3%. Among these, 64% were HIV-infected, confirming that TB is still the most common opportunistic infections associated with HIV. Globally, it is estimated that the largest number of adults having both TB and HIV-infection – approximately 1 million persons – reside in South Africa (15). Between 1997 and 2004, TB was the leading cause of death among all persons (HIV-infected and HIV-uninfected persons) in South Africa (114), which was despite the availability of treatment for TB and later HIV therapy. A study by Osman et al characterised the general morbidity and mortality among adults who had successfully completed TB treatment during the past five years in a high incidence setting in South Africa, and found that among the 51 patients interviewed, almost half reported persistent respiratory symptoms (115). There are many factors contributing to the morbidity and mortality of patients with TB and HIV (116, 117). These factors include side effects of treatment and known adverse events experienced during HIV infection, with anaemia being one of the problems experienced by TB and HIV-infected people. These may negatively affect the quality of life of patients taking these drug combinations.

Apart from side effects, combining HIV and TB therapy may lead to interactions between these therapies. Drug/drug interactions between HIV and TB therapy arise through shared routes of metabolism and are often due to enzyme induction or inhibition (118). The hepatic cytochrome (CYP) P450 system is one important family of enzymes, which is involved in the metabolism of many drugs including the protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), which form part of most ARTs. The NNRTI and PI have proven clinically important drug interactions with the rifamycins, as the

latter are known to be potent inducers of CYP3A4 (119). There are reports that INH inhibits CYP3A4 activity and may therefore its coadministration with drugs that are metabolised by this isoform may result in significant drug interactions (120). Polymorphisms in CYP 2B6 are known to affect the bioavailability of efavirenz, and similarly, *NAT2* also affect the bioavailability of isoniazid (121). Numerous polymorphisms are associated with CYP2B6; the G516T SNP is associated with loss of function leading to high blood concentrations of efavirenz, which may lead to adverse events of the central nervous system (CNS). INH has been reported to mediate the CYP2B6*6 genotype drug-drug interaction between efavirenz and anti-TB therapy through mechanism-based inactivation of CYP2A6 (122, 123), Betrand et al. found that patients carrying the CYP2B6 516TT genotype and *N-acetyltransferase 2* (*NAT2*) slow acetylation phenotype had the lowest efavirenz apparent clearance (124).

There is a relatively high risk of drug-induced hepatoxicity in patients on combined treatment with ARVs and TB drugs, several of which are known to be hepatotoxic. The rate of acetylation of isoniazid, influenced by polymorphisms of the *NAT2* gene, is associated with the risk of hepatoxicity. (60). Slow acetylators having a higher risk of developing side effects like peripheral neuropathy and hepatotoxicity than fast acetylators (35, 125). On the other hand, fast acetylators have a higher risk of developing drug resistance. Genetic differences among individuals can affect ways in which the body responds to drug metabolism, distribution to cells and tissues and elimination from the body (126).

Diagnosis of TB and monitoring of drug adverse reactions is challenging in patients taking both anti-TB and antiretroviral drugs. It is difficult to decide which drug caused the event, since similar metabolism routes are found in some TB and HIV therapies or whether it is because of the illness. Baseline assessments and screening tests are very important to record the status of the patient before starting treatment as this will assist in effective monitoring. A better understanding of the side effects of combining ATT and ART would assist with monitoring of patients. Incorporating HRQOL measurements as part of assessing the treatment response would elicit evidence on treatment outcomes providing better post-treatment information on patients' wellbeing. Different levels of response to treatment may have serious consequences such as infectiousness, increased morbidity, and even mortality.

There is a constant search for appropriate biomarkers for diagnosis and monitoring of TB and HIV treatment. Very few of the thousands of biomarker discovery research yields final products. Gene Xpert MTB/RIF and urine LAM are the two recent products of the research and both are now being used for

diagnosis of TB. At the time of the studies, urine LAM ELISA was one of the biomarkers newly released for the diagnosis of TB, while there was very limited information about QFT-GIT in active TB-HIV-infected persons. We chose to evaluate these biomarkers in two different cohorts; one in smear-negative patients for LAM and the other in smear positive patients for QFT-GIT. We also realised that there was limited information about the NAT2 genotype in the black population of South Africa, especially among the Zulus where there was high prevalence of all forms of TB, including drug resistant forms. We chose to evaluate NAT2 in a PK study being conducted. We added the longitudinal QOL instrument as an additional marker for the same cohort.

1.3 Research question – study hypotheses

How can we best diagnose TB and monitor treatment in patients taking combined TB and HIV therapy?

The study hypotheses are:

1) Polymorphisms in drug metabolising enzymes, specifically *NAT2*, may explain the variations in the experience of adverse events and response to treatment.
2) QFT-GIT is useful to monitor treatment response in TB and HIV co-infected patients receiving combined treatment
3) HRQOL can be a useful tool in assessing the effectiveness of combined TB and HIV therapy.
4) Urine LAM is a useful biomarker for diagnosis of TB in smear-negative individuals and can potentially reduce antibiotic overuse.

1.4 Thesis aim and objectives

The aim of the thesis is to report on the use of a combination of markers for TB diagnosis and treatment monitoring using urine LAM, *NAT2* genotype, QFT-GIT, and HRQOL in TB and HIV co-infected patients.

Objective 1 – To assess polymorphisms of *NAT2* in participants receiving combined TB-HIV therapy.

Sub-objective 1.1- To determine genetic polymorphism frequency in the TB-HIV co-infected isiZulu-speaking black patients.

Sub-objective 1.2 - To evaluate the relationship of genetic polymorphisms with drug levels and inter-individual variability of INH.

Objective 2 - To determine the kinetics of mycobacterial cellular immune responses in TB-HIV patients on combined treatment using an INF-γ release assay.

Objective 3 – To evaluate HRQOL in TB-HIV co-infected patients receiving combined treatment.

Sub-objective 3.1 - To examine changes in the HRQOL of patients receiving TB-HIV co-treatment.

Sub-objective 3.2 - To evaluate whether HRQOL differs in patients receiving TB treatment alone or together with ARV therapy.

Sub-objective 3.3 - To compare changes in the HRQOL of co-infected patients receiving TB-HIV co-treatment with those receiving TB treatment alone.

Objective 4 - To determine the utility of urine LAM in diagnosis of TB in seriously-ill HIV-infected patients with TB.

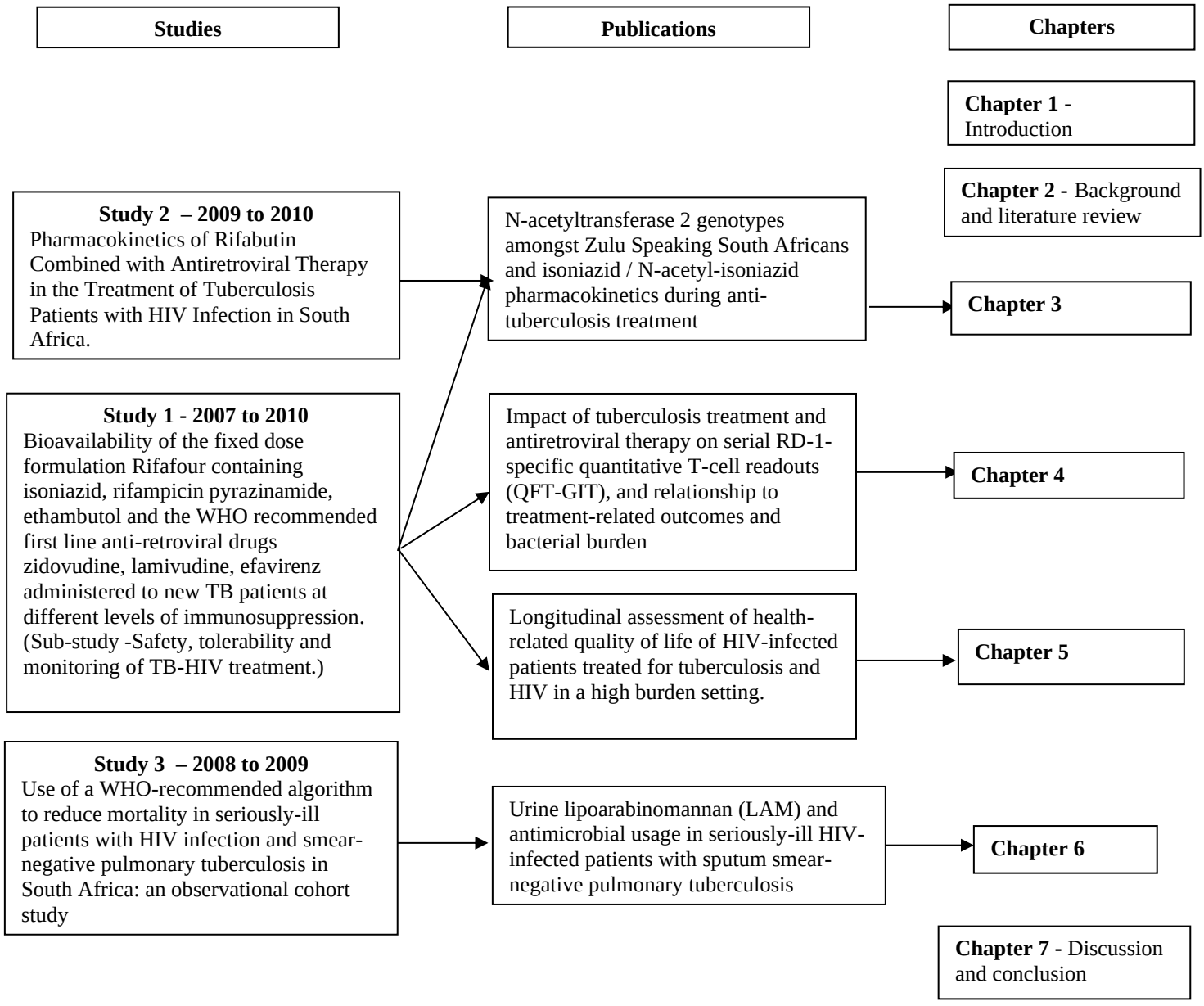
Sub-objective 4.1- To determine the utility of urine LAM for the diagnosis of smear-negative TB.

Sub-objective 4.2- To evaluate the potential utility of urine LAM to reduce antibiotic usage.

1.5 Studies reported in this thesis

In this thesis we report findings of three studies as shown in the Flow chart below.

Figure 1 Flow diagram: Diagnostic and therapeutic biomarker responses in HIV and tuberculosis co-infected patients.



Three studies are reported in this thesis:

Study 1 was a prospective, longitudinal sub-study to evaluate "Bioavailability of the fixed dose formulation Rifafour containing isoniazid, rifampicin pyrazinamide, ethambutol and the WHO recommended first line anti-retroviral drugs zidovudine, lamivudine, efavirenz administered to new TB patients at different levels of immunosuppression." that assessed HRQOL and QFT-GIT kinetics from baseline to months three, six and 12 in TB-HIV co-infected patients with receiving TB treatment and HIV therapy, and HIV patients without TB, as well as assessment of *NAT2* polymorphisms to determine patients' INH acetylation profiles and effect on treatment.

Study 2, "Pharmacokinetics of Rifabutin Combined with Antiretroviral Therapy in the Treatment of Tuberculosis Patients with HIV Infection in South Africa", was a randomised controlled trial of two different rifabutin doses co-administered with lopinavir/ritonavir-based antiretroviral therapy. We used the data collected to investigate isoniazid pharmacokinetics and NAT2 polymorphisms were determined.

The writer of this thesis was not involved in the design and conduct of this study, the isoniazid PK data are analysed and reported in this thesis.

Study 3 was a sub-study conducted as part of a prospective cohort study evaluating the use of a WHO-recommended algorithm to reduce mortality in seriously-ill HIV-infected patients with sputum smear-negative PTB in South Africa.

1.6 Definition of terms

1.6.1 Pharmacogenetics

Pharmacogenetics involves pharmacokinetic and pharmacodynamic studies. Pharmacokinetics describes drug concentration over time, allowing for estimation of clearance, to which drug metabolism contributes. Drug metabolizing enzymes can broadly be classified into two types, phase I (e.g. mixed-function oxidase system, and phase II (e.g. conjugating enzymes) and are located primarily in the liver. NAT2 is a phase II enzyme, wide variation in its expression is associated with highly variable exposures to certain drugs such as isoniazid (127). NAT2 acetylation polymorphism results in segregation of humans into slow, intermediate and rapid acetylator phenotypes. INH is metabolised in the liver and gut mucosa, catalysed by NAT2, resulting in the formation of acetyl-isoniazid (AcINH) and is a highly polymorphic drug (128).

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1.6.2 Interferon-gamma release assays (IGRAs)

IGRAs are tests that can be used as surrogate markers of disease, in this case, TB. These tests are based on antibody recognition of *Mycobacterium tuberculosis (Mtb)* antigen by the body's immune response. T-lymphocytes release IFN-γ when exposed to specific antigens. Specifically for TB, ESAT-6 and CFP-10 are encoded by regions of the *Mtb* genome which are absent from *M. bovis* BCG, *M. avium* and most of the non-tuberculous mycobacteria (129). These antigens have been shown to elicit strong IFN-γ responses from the T-cells of persons infected with *Mtb* but not from the T-cells of those at low risk of infection or those solely BCG-vaccinated (130-132). Berthet et al. found that ESAT-6 and CFP-10 are both secreted and may interact with one another or have a common function in *Mtb* (132). For this reason, the IGRAs have been suggested as being useful for the monitoring of treatment.

1.6.3 Health-related quality of life

The WHO's definition of health is, "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (133, 134). The QOL concept includes multiple factors affecting a person and may include physical well-being, financial, and social functioning. Physical well-being may include mobility and ambulation, ability to do usual activities and experience of pain. Mental health may include cognitive function, emotional health and its limits on functioning. Social well-being may involve abilities to interact with friends and family, intimacy, spirituality, and aspects of the social and physical environment in which the person lives (135). In a clinical setting, Cleary et al. defined QOL as condition-specific symptoms, body discomfort, social and role functioning, the overall perception of health, cognitive status, and general well-being (136). HRQOL has also been defined as how well people are able to perform activities of daily living and their well-being (113).

1.6.4 Urine LAM for TB diagnosis

Currently, it is only through sputum microbiological culture where *Mycobacterium tuberculosis* is recovered that the diagnosis of active TB is definitively made. Diagnosis of and assessment of treatment response of TB in human immunodeficiency virus (HIV) infected patients is difficult and often present with sputum smear-negative results, (smear microscopy detects less than half of TB-HIV co-infected patients)(18), atypical radiographic findings and resemblance to other opportunistic pulmonary infections (137). Thus, the treatment of smear-negative TB patients is often delayed and clinicians may have to resort to other invasive procedures which lead to further delays in diagnosis and treatment. Delays in treatment would increase the morbidity and mortality which is already rated too high. There are few new tests that

have been approved for diagnosis of active TB, these include tests like Gene Xpert MTB/RIF, which has been used in different body fluids such as blood, sputum and urine. Gene Xpert MTB/RIF is approved by WHO for use to diagnose active TB in sputum. Urine LAM has recently been approved in diagnosis of active TB in HIV-infected patients with severe immunosuppression (CD4 count <100 cells/mm^3 (138).

1.7 Justification of the study and outline

Numerous reports indicate the difficulty in diagnosing TB in HIV-infected individuals, especially when they are seriously-ill as often they present with smear-negative disease (18, 139). There is a constant need to test biomarkers for diagnosis and treatment monitoring of TB and HIV co-infected patients (140-143). When an HIV-infected patient is diagnosed with TB, and in most TB programmes, HIV-infected patients need to be started on ART if not already on it. In South Africa, at the time of the study, patients were started on ART only when their CD4 counts had decreased to below 200 cells/mm^3 from 2004 (144). There were questions on the appropriate time to start ART in patients with TB co-infection. The Starting Antiretroviral Therapy at Three Points in Tuberculosis (SAPIT) trial was a pivotal with strong indications that patients could safely take ART with TB treatment (145, 146). In 2010, new guidelines were published, requiring ART to be given to TB patients when their CD4 count was below 350 cells/mm^3 (147). These guidelines were followed by ones increasing the CD4 cut-off point requiring that all TB-HIV co-infected patients must be started on Highly Active Antiretroviral Therapy (HAART) irrespective of CD4 count in 2012 (148). Although the latest guidelines require all patients with active TB should be started on ART, we still find some patients starting ART at very low CD4 count levels and others who stop taking ART for different reasons (149, 150). Accordingly, this raises issues of adverse drug events for each individual drug along with drug-drug interaction between TB and HIV therapy.

Pharmacogenetics deals with some of the issues related to the genetic aspects of drug metabolism and the influence of genetic predisposition on individual drug levels. The influence of NAT2 on isoniazid metabolism and adverse effects is known in different populations. Knowledge of genetic polymorphisms of a population may be useful in determining whether the drug regimen doses are appropriate for the population in question. Adjustment of regimens may be possible, in line with the findings of the genetic results. In the population of choice for the study of NAT2, there is a high relapse rate and investigation of the influence of NAT2 in this population may help understand if there is a relationship. Diagnosis and assessment of TB therapy responses need to be improved as the present methods are sometimes not possible. Most often pulmonary TB patients are unable to produce sputum, especially after a few months

of treatment. Patients with extra-pulmonary TB may need more invasive methods of sputum collection to confirm their diagnosis. There is a need for tests that are non-invasive and tests that give results within a short space of time, possibly within the same day. IGRAs may be useful in this aspect, although they are not recommended in active TB in high prevalence countries.

Since South Africa has one of the highest rates of TB and HIV patients, it is important to have a good and useful system for evaluating patients on combined therapies. The use of quality of life assessments before, during and after treatment may be one of the useful methods. This method is not expensive and can be used as an addition to existing methods (smear and culture).

1.7.1 Sample size and power justification

The sub-studies were conducted from projects with sample size calculated for those objectives. For the study investigating LAM as a diagnostic, assuming a test sensitivity of 50% with at least 50% of probable TB patients meeting the final TB diagnosis, a level of significance of 0.05% and to achieve a margin of error of 10%, we would need a sample size of 96. See table below for the estimates.

Confidence level %	Proportion %	Error margin %	Sample
95	50	10	96
95	60	10	92
95	70	10	81
95	80	10	61

The PK study used for the NAT2, QFT-GIT and QOL evaluations: assuming a difference of 20%, 95% confidence interval and 80% proportion, a sample size of 15 per arm was adequate. We, however, used the existing samples calculated for the original studies without reducing or increasing the sample size because of limited funding.

The next section, Chapter two, deals with the review of the literature, which looks at each concept in the hypothesis, biomarkers of TB diagnosis and monitoring, pharmacogenetics, IGRAs, quality of life and urine LAM. Chapter three covers the *NAT2* polymorphisms , and in Chapter four the results of IGRA are

presented, Chapter five the results of the QOL are presented, in Chapter six covers urine LAM for diagnosis of smear-negative TB. Finally, the discussion and conclusion are presented in Chapter seven.

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Chapter 2

Literature review

2.1 Introduction

This review covers the four areas that are subjects of this thesis; NAT2 polymorphisms for prediction of isoniazid pharmacokinetics variability and adverse events; IGRAs for monitoring of combined TB and HIV therapies in co-infected patients, monitoring treatment response using a QOL instrument in TB and HIV co-infected patients, and finally, urine LAM for smear-negative TB diagnosis.

Early diagnosis and treatment of TB are the necessary components for curbing the spread of the disease. In TB surveys it has been determined that 40 – 79% of the prevalent cases do not report any signs and symptoms of TB (1). By the time an individual experiences signs and symptoms of TB, he/she has possibly infected a number of individuals around him/her (2). HIV is another player in this area, as TB is one of the most common opportunistic infections in people living with HIV (3). Any new diagnostic, to be effective, must be able to diagnose TB in HIV-infected individuals, including those with severe immunosuppression. These individuals are often sputum-scarce, too sick to cough out sputum, or present with smear-negative TB disease, making it difficult to diagnose (4). An ideal diagnostic should also be performed at the point of care. New biomarkers for detection and treatment monitoring are urgently needed to ensure early diagnosis and treatment of active TB, especially in high TB and HIV endemic countries (5, 6). Xpert MTB/RIF is a recent biomarker that effectively diagnoses TB in both HIV-infected and uninfected. Unfortunately, although very sensitive, there are still people that are not diagnosed by this test, and it has not replaced smear and culture, especially in relapse and reinfection TB.

Studies investigating different biomarkers in detecting latent and active disease have been ongoing for some time. Different types of biomarkers include host-derived (immunological) and pathogen-derived biomarkers (7-10). Among these biomarkers are IGRAs, that seemed to be promising for the diagnosis of TB but were found not to be specific for active TB, leading to the WHO cautioning against their use in high endemic countries (11). While IGRAs have been ruled out as a diagnostic for active TB diagnosis, studies have been conducted to investigate their use in TB treatment monitoring (12-14). There are conflicting results in this regard, although it is generally accepted that these should not be used for treatment monitoring. Urine LAM has been investigated for diagnosis of TB, and for a long time, it seemed that urine LAM would not be useful because of its low sensitivity (15-18) but studies investigating its

usefulness in diagnosis of TB in HIV-infected individuals have found its usefulness in severely immunocompromised individuals (19-21).

NAT2 polymorphisms involved in drug metabolisms, IGRAs, QOL and urine LAM, are the key terms for discussion in this chapter. QOL or HRQOL has been studied in HIV-infected individuals and there are few studies reporting the usefulness of QOL as a biomarker for treatment monitoring.

2.2 Definition of key concepts

The biomarker working group defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention(22). Using the term widely, we have included the HRQOL in our definition of biomarker as it has been used as predictive marker of worsening of certain conditions (23). Boxwala et al. defined an adverse event as, "An undesirable event occurring in the course of medical care that produces a measurable change in patient status" (24). The adverse events may be clinical or laboratory parameters and may or may not be related to the treatment. Safety means the level to which side effects to treatment are experienced by patients taking the drugs for TB or HIV therapy or the combined treatment. Furthermore, it may include known or unknown side effects of TB treatment and ART involving different body systems. These may include worsening of the condition, in this instance, TB and HIV. Side effects are any unintended effect of a pharmaceutical product occurring at doses usually used in humans, which are related to the pharmacological properties of the product (25).

TB treatment outcomes, according to the WHO and International Union against TB and Lung Disease (IUATLD) (26) are:

- Cure: a patient who was initially smear-positive and has completed treatment and has two negative smears, one on completion of treatment.
- Treatment completion: an initially sputum smear-positive patient who has completed treatment, has a negative smear at the end of the initial phase and no sputum smear at the end of treatment.
- Treatment loss to follow up: a patient who at any time after registration has not taken treatment for at least two or more consecutive months.
- Failure: an initially smear-positive patient who is smear-positive at five months or more after starting treatment.
- Death: a patient who died while on treatment, regardless of the cause.

- Treatment relapse: TB relapse is defined as a patient who has become (and remained) culture negative while receiving therapy but after completion of therapy becomes:

    1. Culture positive again, or
    2. Has clinical or radiographic deterioration that is consistent with active tuberculosis.

Monitoring involves the perception of the individuals' subjective experiences on how treatment is affecting them. Monitoring clinical characteristics involve laboratory tests, adverse events and serious adverse events reporting. LAM, pharmacogenetics or genetic factors involved in drug metabolisms, IGRAs as well as QOL or HRQOL are the key terms for review in this chapter.

2.3 Literature review

The review of literature will cover the four biomarkers discussed in this study; TB diagnostics, 2.3.1; NAT2 polymorphisms; 2.3.2, interferon-gamma release assays; 2.3.3 quality of life; and 2.3.4. Urine for diagnosis of smear-negative TB.

Biomarkers

Strimbu and Tavel defined the biomarker as objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly (27). The National Institute of Health (NIH) biomarker working group defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (22) and have created a glossary of terms relating to biomarkers. They have defined different biomarkers including diagnostic, monitoring, pharmacodynamic/response, predictive, prognostic, safety, susceptibility/risk biomarkers. A diagnostic biomarker is used to detect or confirm the presence of a disease or condition of interest or to identify individuals with a subtype of the disease. There are different definitions of a biomarker in the literature. A biomarker can be defined as a characteristic that is objectively measured and assessed as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.

According to Selleck, Senthil and Wall, to be truly valuable, "a biomarker must contribute clinically relevant information beyond what is available or provide the same information at a lower cost, either financially or in measurable patient risk" (28). This is true for urine LAM, studies have shown that it

diagnoses hard to diagnose patients with smear-negative and extra-pulmonary TB. It is also cheaper than currently available tests and is a point of care test.

A monitoring biomarker is defined as a characteristic measured serially for assessing status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environment agent. According to Ptolemy and Rifai, in addition to identifying illnesses, biomarkers can be useful as an indicator for clinical or therapeutic intervention efficacy (29), but notes the lengthy process of clinical validation. Califf clarifies that biomarkers should be distinct from direct measures of how a person feels, functions, or survives – a category of measure known as a clinical outcome assessment (COA) (30). COAs can be used to meet standards for regulatory approval of therapeutics, whereas, according to Califf, biomarkers serve a variety of purposes, including linking a measurement to a prediction of COAs. Certain diagnostic biomarkers may be used as monitoring biomarkers as well. It is noted that a diagnostic biomarker may be useful in one set of clinical circumstances while misleading in another context. We note in the case of urine LAM that a negative test is common in HIV-uninfected or in HIV-infected with low levels of immunosuppression.

Pathogen derived biomarkers

Tucci, Gonzalez-Sapienza and Marin conducted a short review on biomarkers derived from M. tuberculosis and their usage in active TB diagnosis (8). They report that diagnostic tests based on detection of LAM in urine were among the first to move from research to commercial stage due to their promising initial results. LAM is one of the successes of a diagnostic biomarker that is derived from *M. tuberculosis*.

2.3.1 Pharmacogenetics and NAT2 polymorphisms

Pharmacogenetics

The concept of genetic predisposition began over 140 years ago through the pharmacogenetics work of Francis Galton and Archibald E. Garrod (31, 32). Pharmacogenetic research emerged as a discipline attempting to understand the hereditary basis for variability in response to therapeutic agents among individuals. The variability in drug response ranges from small to large differences in response among individuals. Variations in the hepatic phase II system cause a wide variation in expression by drugs such as isoniazid and hydralazine (33).

Genetic polymorphism

The term, “genetic polymorphism”, means a difference in deoxyribonucleic acid (DNA) sequence among individuals, groups or populations (34), encompassing monogenic traits that exist in the normal population in at least two phenotypes, neither of which is rare (35). Phenotypes are the visible properties of an organism, which are produced by the interaction between the genotype, or the genetic constitution of an individual, and the environment. Many factors contribute to inter-patient variations in expression of pharmacokinetic and of pharmacodynamic-type polymorphisms. SNP is a source variance in a genome, a single base mutation in DNA (34). SNPs are the most simple forms and most common source of genetic polymorphisms (34). SNPs in a coding region can have two different effects, synonymous, which causes no amino acid change, or non-synonymous, which results in an alteration of the encoded amino acid. The effects of a pharmacogenetic variant on an individual depends on the metabolic pathway of the medication. A loss-of-function polymorphism of the main metabolic pathway of medication, may result in extremely high concentrations of the medication—even into the toxic range. If, on the other hand, the polymorphism affects a minor enzymatic pathway, there may only a negligible change in drug concentrations.

Drug-metabolising enzymes

Genetic polymorphisms in drug metabolism are quite common among the population because of differences intrinsic (physiologic) and extrinsic (cultural, behavioral and environmental characteristics of people (36). These factors may cause mutations in the genes for drug-metabolising enzymes (DMEs) (37). The mutation can cause higher or lower enzyme activity or lead to the partial or total absence of an enzyme protein. Polymorphisms have been defined for numerous DMEs, and many of these were identified after an adverse drug reaction occurred from a standard dose of medication. These adverse reactions then led to the evaluation of the association between decreased drug clearance and decreased activity of DMEs.

For certain DMEs, a constant percentage of the population lacks or has greatly reduced enzyme activity. Based on these differences, the population can be subdivided into poor metabolisers and extensive metabolisers (38). Genetic polymorphism exists when an inherited trait is maintained in the population because the gene controlling the trait is present with a frequency greater than 1% (39). Genetic variants of *NAT2* are amongst the better-studied polymorphisms (40, 41). *NAT2* polymorphism was discovered more than 50 years ago using INH as the substrate probe. INH is bactericidal against actively growing *Mtb* and is bacteriostatic against non-replicating organisms (42, 43). It acts through inhibition of oxygen-dependent synthetic pathways of mycolic acid, an important constituent of mycobacterial cell walls. Significant

interpatient variability in serum concentrations of INH was recognised early, generally occurring hours after an oral dose (44, 45).

NAT2 polymorphisms

NAT2 activity is reported to be highest in the liver and gastrointestinal tract (46). According to available literature, drugs such as isoniazid and sulfamethazine were termed polymorphic substrates for N-acetylation as a result of the observation that they divided the population into rapid and slow acetylator phenotypes, while other drugs yielded normal distribution (35, 47, 48). Parkin et al. found that isoniazid presented a trimodal distribution in the population, rapid, intermediate and slow acetylation (49). Whether the distribution is bimodal or trimodal, its relationship to treatment effect would not change.

The reference *NAT2**4 gene is associated with rapid acetylator phenotype activity and is referred to as the "wild type" (35, 40). The most commonly investigated SNPs are 191, 282, 341, 411, 590, 803 and 857. Two are synonymous or silent (C282T and C481T), the substitution causing no amino acid change to the protein it produces, whereas the remaining four variants are non-synonymous, the substitution resulting in an alteration of the encoded amino acid (34) (T341C, G590A, A803G and G857A). G191A was not investigated in this study.

NAT2 genotype and phenotype inference

Different methods are used to infer *NAT2* genotype and phenotype of individuals. Determination of *NAT2* SNPs has been done using polymerase chain reaction (PCR) restriction fragment length polymorphism and sequencing techniques (50-52). Methods using gel electrophoresis and microarray systems (53), SNP specific PCR primers and fluorogenic probes as detailed by Doll et al. have also been used (54). More possibilities become available with technological advances (55, 56), especially with new sequencing techniques. A decision on which method to use is based on the cost and numbers. Genotype scoring often used, is according to the nomenclature by Vatsis and co-workers (57) but recently computational methods are being used (58-61). Different methods come with differences in data quality (62), and investigators have to make choices on which methods would work best for their study. The use of Tag SNP (A SNP in strong linkage disequilibrium with a set or a copy number variant) has been described as a good representatives of the rest of the SNPs, but still needs validation (63, 64).

Caffeine is most commonly used probe drug for NAT2 phenotyping by measurement of urinary molar ratio of the caffeine metabolites (65). Phenotype inference from isoniazid pharmacokinetic sampling has been conducted using different methods. Investigators have used any one or a combination of elimination half-life of INH (66, 67), INH plasma levels at six hours after drug intake, inactivation index (I_3) (68), and molar acetylation ratio (69). Hitchings et al. found that using the 3-hour isoniazid AcINH/INH was better than the elimination half-life method (70). There is no gold standard for the classification of phenotype. Investigators use different criteria to classify INH phenotype, although the inactivation index (metabolic ratio) is the most commonly used (71-73). Different studies have used different SNP combinations to infer phenotype. Because of the high rate of admixture in South Africa, using either a 2 or 4 genotype panel to infer phenotype may not be adequate. The accuracy of the SNP panels differ from population to population, where a 3-SNP panel of 481>T, 590>A and 857>A was 100% sensitive in inferring phenotype for Guarani and Japanese, 79.4% for Mozambicans (79).

Adverse events of anti-TB treatment and antiretroviral therapy

The achievement of therapeutic drug levels of ATT and ART is necessary to decrease the morbidity and mortality related to TB and HIV. For the absorption, distribution, metabolism, elimination and action of these drugs to be understood, the influence of pharmacogenetics needs understanding (74). Most patients will tolerate ATT well, experiencing only minimal adverse events, as shown in studies although, some patients may experience severe incapacities because of adverse events of either ATT, ARV therapy or combined therapy (75, 76). Their results showed that the risk of adverse reactions is significantly higher in HIV-infected patients. Multidrug therapy of any kind can be complicated by overlapping toxicity profiles, such that it may be difficult to determine which of several agents being used caused a specific adverse effect (77).

Guidelines issued by various agencies provide different initiation recommendations according to resource availability (78-80). Consequently, the decision to initiate ART in co-infected patients has always been made on clinical grounds and a case-by-case basis, while until recently, all patients in resource-poor settings received treatment only when their CD4 count dropped to below 200. A decision on when to start ART in an HIV-infected, asymptomatic patient has been controversial for a long time. There is enough data from observational and randomised clinical studies that support the idea that most, if not all, individuals with HIV would benefit from treatment (81-83). The most benefits of ART in TB patients results from a gradual restoration of *Mtb*-specific CD4 cell immune responses (84).

NAT2 acetylation and isoniazid side effects have been investigated extensively (85-90), and early studies reported peripheral neuropathy and hepatoxicity and its relation to slow isoniazid acetylation. Since not only INH but pyrazinamide (PZA) and rifampicin have been responsible for hepatotoxicity, patients on ATT are more prone to hepatic side effects (91-93). Also, certain ARVs have similar side effects, making concomitant therapy of ATT and ARV leading to increased side effects (88, 91, 94).

2.3.2 Interferon-gamma release assays

There have been suggestions that IGRAs can be used as biomarkers to monitor TB treatment responses (95, 96). Studies conducted to examine the effect of active TB treatment on INF-γ responses have had inconsistent results. Some studies have shown declining responses (97) while others have shown unchanging, fluctuating or increasing responses post-treatment (98-101). This variation may be because of differences in methodology and types of tests used.

Mtb initially infects macrophages, and cellular immunity plays a vital role in the healing process of TB (102). Macrophages produce toxic radicals and hydrolytic enzymes to defend the body against *Mtb*. However, the *Mtb* has a rich lipid outer capsule which protects it from the body's defences. Macrophages, after activation, possess potent antimicrobial activity which can kill bacilli and arrest TB infection, which is not entirely eliminated easily. Cytolytic T lymphocytes may be used as well in the destruction of the infected cells (103, 104). Helper T-cells (Th) are needed to recruit and activate new monocytes and macrophages to the tubercle. CD4+ T-cells are divided into Th1 and Th2 subsets, depending on the type of cytokines produced. Th1 cells produce the cytokines IFN-γ and interleukin (IL), which are important for the activation of any antimicrobial activities and essential for delayed type hypersensitivity (DTH) response. IFN-γ specifically activates macrophages and stimulate them to ingest and kill mycobacteria more effectively.

The induction of INF-γ has been shown to be essential for individual protection against TB (79, 105). Surcel et al. investigated Th1/Th2-like cell profiles in patients with active TB (106). They studied T-lymphocyte activation by analysing IL-4 and INF-γ production at single-cell level using Enzyme-linked Immunosorbent Spot (ELISPOT) assays in peripheral blood mononuclear cells (PBMC) stimulated with individual mycobacterial antigens. They found that the average numbers of cytokine secreting cells were generally elevated in TB patients following stimulation with murine tracheal surface epithelial (MTSE)

containing a wide range of antigens. The numbers of IL-4-producing cells were significantly elevated in PBMC from TB patients compared with controls in response to 38,000 MW antigen, while the 19,000 molecular weight (MW) antigen-induced more prominent activation of INF-γ-producing cells in TB patients compared to controls. They found that the number of INF-γ secreting cells did not differ significantly between patients and controls. Increased INF-γ secretion was found slightly more frequently in patients than in controls after three days of antigen stimulation.

The use of surrogate endpoints has been widely examined in HIV and acquired immunodeficiency syndrome (AIDS) compared to in any other disease (10). Surrogate markers for TB and HIV/AIDS are essential for the clinical evaluation of the extent of the disease as well as response to treatment and vaccines. According to Lawn et al., findings that patients are still prone to TB while on antiretroviral treatment, is an indication that complete restoration of cellular immunity against TB may not be possible (107). Of interest is, what the effect on the immunity of starting antiretroviral treatment together with ATT would be in co-infected patients.

A study by Schluger et al. was conducted to assess the restoration of immune responses to TB, as manifested by secretion of T-helper type 1 cytokines (IFN-γ, IL-12, and IL-2) and T helper type 2 cytokines (IL-10), in HIV-infected patients on ART (108). Included in the study were ten antiretroviral treatment naïve HIV-infected patients, who were about to start ART for clinical indications and 11 HIV-negative healthy participants. They assessed the T-cell proliferation and cytokine production after administration of ART to patients with HIV infection. All patients had a baseline negative tuberculin skin test (TST) and were anergic. Blood was drawn at two-monthly intervals for 12 months after the start of treatment. This study demonstrated that *mycobacterial* immune responses are restored to some degree, although delayed compared to HIV viral load suppression and CD4+ T-cell increase. Also, the restoration was found to be submaximal when compared with responses seen in peripheral mononuclear cells (PBMC) obtained from healthy volunteers. IFN-γ production reached levels seen in healthy volunteers only after several months of ART.

Subramanyan et al. conducted a study to test the hypothesis that HIV infection brings about an alteration in the host immune response to TB (109). Levels of the proinflammatory cytokine IFN-γ and its regulatory cytokines (IL-12, IL-18 and IL-10) in plasma were measured in TB (HIV-uninfected and -infected) patients and their controls. Then stimulation of PBMC by mycobacterial antigens followed. They found

that TB patients had higher IFN-γ than non-TB patients, with TB/HIV co-infected patients having less than TB non-HIV patients. Plasma levels of IFN-γ were found to be significantly higher in HIV-infected TB patients with low CD4 counts (<200 cells/mm^3) than in HIV-uninfected TB patients as well as in HIV-infected TB patients with higher CD4 counts. The presence of significantly higher levels of IFN-γ in individuals with low CD4 counts suggested a greater degree of immune activation.

In another study, Wu et al. compared the INF-γ response between TB patients and non-tubercular pneumonia patients (110). INF-γ responses were measured using an enzyme-linked immunosorbent assay (ELISA) test. Results showed that patients with TB had less INF-γ expression than those with pneumonia. They also looked at other co-morbidities and how these affected INF-γ production. In vitro INF-γ response did not differ between patients with and without other co-morbidities, such as liver cirrhosis, bronchiectasis and diabetes mellitus, although in other studies, lower INF-γ responses were found in patients with diabetes mellitus. The researchers concluded that TB was the only independent factor associated with INF-γ response in patients with lung infection indicating that INF-γ response was principally affected by *Mtb* infection and not by other co-morbidities.

Pai et al., in a study to evaluate the INF-γ responses in health care workers diagnosed and treated for latent TB, found no significant changes in the responses to treatment in subjects who took medication. The study was limited by a small sample of ten subjects who took treatment. Another explanation of the persistent INF-γ levels would be a persistent infection and or re-infection (unproven). Authors recommended a follow up longer than six months (111). A similar study in Japan showed a decline of INF-γ levels soon after treatment (112, 113). Chee et al. studied the effect of TB treatment on IGRA responses measured by QFT-GIT and T-SPOT.*TB* (97). There were significant declines in the positivity rates and qualitative results of both IGRAs with treatment. They reported significant test reversion rates for both tests, a substantial proportion of patients, however, remained positive after TB treatment.

To evaluate the feasibility of ELISA assay for INF-γ during HIV infection, Goletti et al. enrolled 16 patients with active TB disease (smear- and culture-positive): ten HIV-infected and six HIV-uninfected patients (114, 115). They included six HIV-infected patients with opportunistic infections other than TB and four HIV-negative individuals as controls. ELISPOT or ELISA for IFN-γ was performed in cells stimulated with ESAT-6 peptides and correlated with response to HIV-uninfected and recall antigens. At the time of TB diagnosis, seven HIV-infected individuals showed an *in vitro* response to purified protein

derivative (PPD) all of whom responded to ESAT-6 peptides. This response decreased over time and became negative after three months of therapy when patients were sputum TB-Ribonucleic acid (RNA) and culture-negative. At baseline no response to antigens tested was observed in five of HIV-TB patients analysed; although at follow-up four out of five showed induction of response of at least two of the recall antigens tested. Within TB-HIV-negative patients, five responded to PPD, and four out of five responded to ESAT-6 peptides. The response to stimulation decreased over time. None of the TB-negative controls, either HIV-infected or non-infected healthy controls responded to ESAT-6 although some were PPD positive. It was concluded that it is possible to perform an immune diagnosis of TB based on the detection of ESAT-6 peptide-specific IFN-γ secreting CD4 T-cells in HIV-infected individuals, even in patients with low CD4 cell counts. Since this response decreases over time and correlates with successful ATT and to a restored induction of recall antigen response, it is possible that this test may be used to monitor TB treatment response. Although findings from these studies vary greatly, they show that it might be worthwhile to investigate the role of IFN-γ in monitoring TB-HIV treatment.

A recent study evaluated the diagnostic performance of QFT-Plus and compared it with the QFT-GIT test in Korean patients with active TB and healthy controls including subjects with latent TB infection. This test includes a TB2 antigen tube with peptides that may stimulate CD8+ T cells. They found that both tests had the same sensitivity of 93.9%, but the specificity was higher in the QFT-GIT test. The QFT-Plus test did not clearly discriminate between active and latent TB although IFN-γ concentrations were lower in those with latent TB (116). This study concluded that the tests had excellent agreement and further surveys are needed to evaluate the role of QFT-Plus to determine patients with different stages of TB infection or to monitor treatment effect. In addition, this study did not include HIV-infected patients; therefore, it would be beneficial to evaluate this test in such patients. Another study evaluated the effect of HIV on QFT-Plus test in HIV-infected patients with active and latent TB infection in Zambia (117). They found that the test had similar sensitivity in both HIV-infected and uninfected patients, and that CD4 count did not influence the IFN-γ values in active and latent TB patients.

2.3.3 Quality of life

The definition of health by the WHO in their Constitution of the WHO, states, “Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity” (118),

this is consistent with other definitions. There are multiple definitions for the quality of life, each one depending on the discipline or disease for which the QOL is being measured. The WHO's definition of Quality of Life is, "individuals' perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns. It is a broad-ranging concept affected in a complex way by the person's physical health, psychological state, level of independence, social relationships, personal beliefs and their relationship to salient features of their environment" (119). However, there is a general agreement that QOL, especially HRQOL, includes the physical, emotional, cultural/social, and emotional aspects of a person, among other things.

The term, Health-related quality of life, was proposed by Kaplan and Bush to distinguish between health effects and other factors influencing a subject's perception of his/her health (120). Different studies have investigated QOL in participants receiving treatment for TB or those receiving treatment for HIV (121-127), but studies investigating the QOL of patients co-infected with TB and HIV and receiving concomitant therapy are few and far between (128-130).

QOL and tuberculosis

The possibility of problems related to side effects when receiving TB treatment (131, 132), may be worsened by concomitant HIV therapy, thereby affecting QOL. A comprehensive assessment of patients' health status is essential to consider the overall impact of TB on health and what perception the patient has of his/her wellbeing, as well as routine clinical, radiological and bacteriological assessments (133). According to Dhuria et al., this can be performed by measuring the QOL that has several dimensions (134). Carr et al. discuss the determination of QOL through expectations or experience (135) relating to HQOL as the "gap between our expectations of health and our experience of it". Because of different expectations, people may adapt to the change in their health by altering expectations. This may reduce the minimal important difference (MID), defined by Jaeschke et al. as, the smallest difference in score in the domain of interest that patients perceive as a change in score that mandate a change in the patient's management (136). The individual is deemed the main person reporting his/her subjective experience. They concluded that it was likely that patients have different standards about the changes in symptoms that they view as important or trivial. When conducting research on HRQOL, it would be important to keep this in mind.

There are several reviews of the literature on QOL in patients with TB. The review by Kastien-Hilka et al found that TB and HIV therapy affected the QOL of patients negatively, including adherence to treatment(137). Another review by Bauer et al reported on the QOL of active TB and latent TB infection (LTBI) using different QOL instruments and found that patients with active TB had poorer QOL than those with LTBI(138).

There are two different types of QOL instruments, disease-specific and generic instruments. The generic instruments are general and may miss disease traits in measuring HRQOL, while disease-specific instruments are more focused on the specific disease symptoms and effects. Among the generic instruments are the self-administered instruments such as the sickness impact profile (SIP, and the Medical Outcomes Study Short Torm-36 (MOS). The SIP, with 136 questions, was developed in 1976, and the MOS, developed in 1990, has different versions including the SF-36 which is the most widely used and well known. These instruments have demonstrated reliability and validity in HIV-infected patients. Table 1 gives 12 different instruments, what they measure and advantages and disadvantages in co-infected HIV and TB patients. In a review by Guo et al., SF-36 was the most used HRQOL instrument among TB patients (126). In addition, the Linear Analogue Self-Assessment (LASA) QOL; the Nottingham Health Profile (NHP); the McMaster Health Index Questionnaire (MHIQ); the Cooperative Information Project (COOP); the WHO Quality of Life (WHO-QOL); the Health Utility Index (HUI); and the Reintegration to Normal Living Index (RNLI), are some of the available generic QOL instruments.

Disease-specific instruments for TB are not easily available. Only the St George's Respiratory Questionnaire, a specific questionnaire for respiratory diseases is more widely known. As a standardised, self-administered questionnaire, it is used to measure impaired health and perceived HRQOL in lung disease (139). This instrument consists of 76 items, with a symptom, activity, impact and a total score. The 'symptoms score' assesses the patients' perception of their recent (four weeks) respiratory problems; the 'activity' score measures the individuals' current (in)ability to perform daily physical activity; the 'impacts' score evaluates the whole range of (in)abilities the individuals' currently experience in their life because of respiratory problems, and the 'total' score sums and weighs all the former components.

Dhingra and Rajpal measured HRQL with the DR-12, a new TB-specific instrument, which was developed in India and first published in 2003 (133, 140). Composed of 12 items, seven cover TB symptoms (i.e., cough and sputum, haemoptysis, fever, breathlessness, chest pain, anorexia, and weight loss) and five

relate to socio-psychological and exercise adaptation (i.e., emotional symptoms/depression, interest in work, household activities, exercise activities, and social activities). All 12 response options are presented on a scale of three points with equal weights given to each item when calculating scores.

Table 1: Generic Measures of Health-Related Quality of Life

Instrument	Date developed	Dimensions examined	Length; time to complete	Applicable diseases	Advantages	Disadvantages
SIP	1976	Physical: ambulation, mobility, body care Psychosocial: social interaction, communication, alertness, emotional behaviour Other: sleep/rest, eating, work, home management, recreation and pastimes.	136 items; ≈20 min Self-administered or interviewer	After ICU, Glaucoma, Muscle weakness, end-stage renal disease	Results can be presented as subscale and summary scores; no floor effects	Not HIV or TB-specific; takes longer to administer; emphasis on physical dysfunction
QWB	1980+	Self-care, mobility, institutionalisation, social activities, reports of symptoms and problems (physical and mental) (Formerly Index of Well-being scale).	50 items; ≈20 min Interviewer; self-administered version	Congestive cardiac failure, Postmenopausal and HIV	Can be used to calculate cost-utility	Not HIV or TB-specific; takes longer to administer; single score only
MOS SF-36	1990	Physical functioning, role limitations caused by physical problems, social functioning, body pain, general mental health, role limitations caused by emotional problems, vitality, general health perceptions.	36 items; 10 min Self-administered	TB, HIV	Culturally adapted and translated into > 50 languages	Not HIV or TB-specific
LASA	1980+	Energy level, daily activity, overall QOL.	3 items; 1–2 min Self-administered Also known as the visual analogue scale	Cancer	Short administration time; easy to administer	Not HIV or TB-specific; not as reliable as multi-item measures; may not be truly linear

NHP	1970+	6 domains of experience: pain, physical mobility, sleep, emotional reactions, energy, social isolation; 7 domains of daily life: employment, household work, relationships, personal life, sex, hobbies, vacations.	45 items; 5–15 min Self-administered	General	Evaluates areas pertinent to HIV disease	Not HIV or TB-specific; items negatively worded
Spitzer QOL Index	1980+	Activity, daily living, health, support, outlook on life.	5 items; 10 min Self-administered	General	Relatively short administration time	Not HIV-specific; questionable reliability and sensitivity in HIV patients
MHIQ	1970+	Physical: mobility, self-care, communication, global physical functioning Social: general well-being, work/social role performance, social support and participation, global social functioning.	≈ 59 items; 20 min Self-administered or interviewer	General, HIV	Has been used in a variety of disease states and settings	Not HIV-specific; takes longer to administer; limited reliability
COOP Charts	1987	Physical condition, emotional condition, daily work, social activities, overall condition, change in condition, pain, general HRQOL.	9 items; 5 min Interviewer	General, HIV	Short administration time; easy to administer to patients with limited education	Not HIV-specific

WHO-QOL, WHOQOL-100 and BREF	1994	The WHOQOL-100 produces scores relating to particular facets of quality of life (e.g. positive feelings, social support, financial resources), scores relating to larger domains (e.g. physical, psychological, social relationships) and a score relating to overall quality of life and general health.	Self-administered if respondents have sufficient ability: otherwise, interviewer-assisted or interview-administered forms should be used	TB, HIV	BREF is easy to administer	Not TB specific
Euro-QOL	1990 revised 1993	A standardised, widely validated and tested, instrument that provides a simple descriptive 5-dimensional profile (EQ-5D), a utility-based index of health status, and the EQ-Visual Analogue Scale (EQ-VAS). The EQ-5D descriptive system defines health in terms of five domains: mobility, self-care, usual activities, pain or discomfort and anxiety or depression.	Self-administered	TB, HIV		Not TB specific
RNLI	1980+	The RNLI contains 11 items, on which respondents rate their satisfaction of their physical, emotional and social lives on 100 mm visual analogue scales, where '0' means 'does not describe my situation' and '100' means 'describes my situation'. For the total scores, items scores are summed and averaged. Higher scores represent better reintegration.	Self-administered	General, HIV		Not TB specific
HUI	1980+	The HUI3 system comprises eight attributes with five to six levels per attribute. The McMaster HUI 2 and 3	Self-administered	General, HIV		Not TB specific

		are multi-attribute health classification systems, for which multi-attribute preference functions have been developed in Canada. They provide a comprehensive instrument for use in economic evaluation and population health survey studies.				

SIP: sickness impact profile; QWB: quality of well-being scale; MOS SF-36: medical outcomes study short form-36; LASA: linear analogue self-assessment; QOL: quality of life; NHP: Nottingham health profile; MHIQ: McMaster health index questionnaire; COOP: cooperative information project; HRQOL: health-related quality of life. WHO-QOL: World Health Organization, quality of life; HUI: health utility index; RNLI: reintegration to normal living index

HIV/AIDS HRQOL instruments

The HIV-QOL Questionnaire (HIV-QL31) was designed to assess the effects of clinical interventions in the field of clinical and public research in the HIV-infected (141). The HIV-QL31 had 31 items that would enable the measurement of the impact of HIV infection on the lives of individuals from their subjective view.

Davis and Pathak evaluated four HIV-specific QOL instruments for their psychometric properties (142). They chose the HIV/AIDS Quality of Life (HAT-QOL), a 42-item self-administered instrument based on patient-reported concerns; the Medical Outcomes Study HIV (MOS-HIV); Functional Assessment of HIV Infection (FAHI) and the HIV Overview Problems Evaluation System (HOPES). They used criteria that included administration, content, depth, reliability, validity, and responsiveness. They could not decide on the best instrument to use for measuring QOL in HIV-infected patients. However, the FAHI instrument has been more widely used than the other three. The FAHI was originally developed using the Functional Assessment of Cancer Therapy, which measures generic and disease-specific HRQOL for patients with such chronic diseases as cancer (143), and later adapted to include HIV/AIDS. This instrument is discussed below.

Other disease-specific instruments are, the General Health Self-Assessment (GHSA); the AIDS Health Assessment Questionnaire (AIDS-HAQ) and Multidimensional Quality of Life Questionnaire for HIV/AIDS (MQOL-HIV). The FAHI questionnaire has already been translated into isiZulu which is the language used by most of the study population. This instrument measures HRQOL specifically in HIV-infected individuals.

FAHI instrument

The FAHI evaluates physical well-being, functional and global well-being, emotional well-being/living with HIV, social well-being, and cognitive functioning, yielding a total score and individual subscale scores. The FAHI has excellent psychometric properties (144). Although it has not been used extensively, it has been used to demonstrate significant improvement in HRQOL following treatment of anaemia with recombinant human erythropoietin (epoetin alfa) in HIV-infected patients (145), and effects of etravirine versus placebo (146).

The FAHI, an HIV-specific tool, was originally developed using the Functional Assessment of Cancer Therapy (FACT), which measures generic and disease-specific HRQOL for patients with chronic diseases such as cancer. To better capture aspects important to the HIV-infected population, a revised version comprising 27 items from the original core instrument and 17 new items reflecting specific HIV/AIDS concerns was developed. Therefore, the FAHI contains 44 items and can be used for self-administration. The FAHI evaluates cognitive functioning, social, physical, functional and global well-being and emotional well-being/living with HIV. The FAHI is available in ten languages, including isiZulu, the language mostly used among the study population in KwaZulu-Natal.

Table 2: Summary of FAHI instrument items

Item title	No. of items	Meaning Low score	Meaning High Score	Total Score
Physical well-being	13	positive	adverse	52
Emotional well-being	10	positive	adverse	40
Functioning and global well-being	13	adverse	positive	52
Social well-being	8	adverse	positive	32
Cognitive functioning	3	adverse	positive	12
Total	47			182

2.3.3.1 Adverse events and QOL

Safety of treatment has been defined by the number and severity of adverse events experienced by patients taking the treatment concerned. Tolerability has been defined by the patients deciding to discontinue

treatment because of not being able to handle the adverse effects of treatment. If adverse events of treatment are so serious that the patient should stop treatment, it can be safely assumed that the QOL of that patient is affected. Multidrug therapy of any kind can be complicated by overlapping toxicity profiles, such that it may be difficult to determine which of several agents being used caused a specific adverse effect (77). TB therapy-specific adverse events include gastrointestinal, liver, musculoskeletal and dermatological side effects. In a study by Shargie et al. on smear-positive patients, only 6.7% of defaulters were related to adverse side effects (147). While in a study by Breen et al. to assess the frequency of serious adverse events (SAEs) and treatment interruption, SAEs were recorded in 40% HIV-infected and 26% HIV-uninfected individuals (76). The most common SAEs were peripheral neuropathy and persistent vomiting among the co-infected patients, although ATT interruption was similar in the two groups. In most patients, this was because of hepatotoxicity. In a study by Miller et al. on reasons for default in South African patients taking ARVs, adverse events had little influence in patients' decisions to stop treatment, while transport costs and time needed for treatment were the most common reasons (148).

In Table 3, side effects and drugs that potentially cause these are shown.

Table 3: Side effects of anti-tuberculosis treatment and antiretroviral therapy

Side effect	**Possible causes**	
	Anti-tuberculosis drugs	**Antiretroviral drugs**
Skin rash	Pyrazinamide, rifampicin, rifabutin, isoniazid	Nevirapine, delavirdine, efavirenz, abacavir
Nausea, vomiting	Pyrazinamide, rifampicin, rifabutin, isoniazid	Zidovudine, ritonavir, amprenavir, indinavir
Hepatitis	Pyrazinamide, rifampicin, rifabutin, isoniazid	Nevirapine, HIV-1 protease inhibitors, immune reconstitution after starting antiretroviral therapy among patients with chronic viral hepatitis
Leukopenia, anaemia	Rifabutin, rifampicin	Zidovudine

In studies, it has been shown that most patients will tolerate ATT well, experiencing only minimal adverse events, however, some patients may experience severe incapacities as a result of adverse events of either ATT, ARV therapy or combined therapy (149, 150). Yee et al. found that the incidence of pyrazinamide-induced hepatotoxicity and rash during treatment for active TB was substantially higher than with the other first-line anti-TB drugs and higher than previously recognised (151).

In a prospective study, Lanternier et al. compared the incidence of adverse events related to ATT in HIV-seropositive and seronegative patients (152). Their results showed that the risk of adverse reactions is significantly higher in HIV-infected patients. According to Marra et al., patients mostly complained about side effects related to specific symptoms that were thought to be related to taking specific drug regimens (127). They found that most common complaints were related to gastrointestinal disturbances (nausea, vomiting and diarrhoea) and itchiness caused by INH. Just as side effects of ATT have been reported as responsible for non-adherence to therapy, the same has been reported of HIV therapy (153, 154).

Deribew et al. studied the impact on QOL of TB-HIV co-infection (130, 155). They found that co-infected patients had significantly lower QOL (WHOQOL-HIV) in all domains than HIV patients without TB. However, there was no mention of adverse drug effects. Depression was the main reason for decreased QOL. Duyan et al. reported social factors as the most common reason for lower QOL, adverse effects of TB were reported to be the greatest in poor people (156). According to Chamla, the associated factors of QOL were age, sex, anaemia, white blood cells and the number of symptoms. There was no mention of side effects in this study.

2.3.3.2 Hepatotoxicity

Both anti-TB drugs and ARVs cause hepatic side effects in varying degrees. Besides the gastrointestinal side effects, hepatotoxicity seems to be the most common adverse side effect of both therapies. Sharma et al. 2004, conducted a study evaluating the clinical and immunogenetic risk factors for the development of hepatotoxicity during ATT treatment, and found that the patients that developed hepatotoxicity were comparatively older, had lower pre-treatment serum albumin, and a higher frequency of radiographically moderately/far advanced disease than the patients who did not (92).

2.3.3.3 Impaired lung function and QOL

In a study conducted in South Africa by Plit et al., new cases of pulmonary TB were studied prospectively over six months to confirm the hypothesis that treatment improves lung function despite the development of residual fibrosis (157). These researchers found that over half the patients had residual respiratory function abnormalities at the end of treatment. Pulmonary TB (PTB) has been mentioned as one of the contributing factors of chronic lung disease which is associated with cardiovascular, respiratory and all-cause mortality (158, 159). Maguire et al. conducted a study to quantify the extent to which PTB

contributes to permanent lung damage and its influence on exercise tolerance and QOL in a region with a high number of cases of PTB and chronic lung dysfunction (160). This study concluded that while QOL and exercise tolerance appeared to improve, longer-term clinically significant abnormalities in lung function were observed. Moderate reduction in lung function and exercise tolerance attributable to PTB in individuals represent a high burden of respiratory morbidity in high-burden areas. The factors discussed above indicate that evaluating HRQOL in TB-HIV co-infected patients is important to understand how patients view of their condition and may assist the clinical staff in prescribing relevant interventions for the patients.

2.3.4 TB diagnosis using urine lipoarabinomannan

TB diagnosis remains a problem for every clinician, in spite of decades of advances in clinical medicine. First there has to be clinical suspicion of disease, usually based on the patient's report of signs and symptoms. Unfortunately, the signs and symptoms are not specific for active TB as they may be present in other conditions. The age-old use of chest radiography and sputum examination has its own problems, especially where there is co-infection with HIV. According to Shluger and Rom, upon suspicion of TB a chest radiography and sputum examination for mycobacteria, but they continue to say that the radiographic picture may be atypical for patients co-infected with HIV, with some being normal (161). The search for a TB diagnostic is ongoing and an ideal diagnostic is one that can be used at point of care, is easy to use and gives results immediately (162) and is not dependent on sputum production. There have been advances since then, leading to newer diagnostics such as Xpert MTB/RIF and urine LAM. The advantage of urine LAM over Xpert MTB/RIF is that it is easier to obtain a urine rather than a sputum specimen. Although Xpert MTB/RIF has been tested in other body fluids such as urine, blood and cerebrospinal fluids (163-165), which showed that Xpert MTB/RIF can be useful for the diagnosis of extrapulmonary TB. However, some samples are not easy to get as they need invasive procedures. Urine is the easiest to obtain and although Xpert can detect TB in urine, the LAM test can do that faster using the strip test (166).

As a result of the difficulty in diagnosis of smear-negative and suspected extra-pulmonary TB, often these patients are started on antibiotics and observed for responses. If there is no response, ATT is then started while waiting for sputum culture results. In 2007, the WHO released new guidelines on the management of smear-negative TB patients. These guidelines allow for start of ATT within 3 days of admission in patients who fulfil the criteria for smear-negative pulmonary TB (167). However, several studies reviewing the use of the guidelines have found that they are not followed as they should (168) and there

some limitations for low income countries where culture processing is still unsafe (169). In addition, culture results can take up to 6 weeks to be available, or may be contaminated. Several studies have been conducted to evaluate the use of urine LAM to diagnose TB in seriously-ill individuals with low CD4 counts. Although the sensitivity varies with the level of immunosuppression, a new test from Fujifilm promises to have improved sensitivity while maintaining specificity (170).

Recently, urine diagnosis of TB using LAM has been advocated and WHO guidelines developed for its use in HIV-infected individuals with severe immunosuppression (171). Urine LAM is a test based on the detection of mycobacterial LAM antigen. LAM is one of the lipopolysaccharide components of the cell wall of mycobacterium tuberculosis (172), some studies have explored the detection of LAM in serological fluids and a number of studies have explored the detection of this antigen within blood, sputum, pleural fluid and urine as a means for TB diagnosis (15, 163, 173).

The review by Minion et al found that urine LAM sensitivity was 3–53% higher in HIV-positive than HIV-negative subgroups; sensitivity was highest with advanced immunosuppression (18). This finding has been further confirmed in a review by Lawn, suggesting that the potential utility of LAM ELISAs are at point of care, within an outpatient setting or hospital ward (174). A further benefit of the urine LAM test is that it can be used by health care workers without any laboratory training nor diagnostic hard-ware or electricity. A review by Gupta-Wright found that a positive urine LAM test was associated with increased mortality in HIV-TB patients (175). In a study by Peter et al, LAM identified a different subset of patients with more advanced immunosuppression and greater disease severity (176). Urine LAM test has been research extensively there is consensus in its usefulness in HIV-infected patients with severe immunosuppression (21, 166, 176-178).

This chapter has dealt with the literature on a combination of biomarkers, *NAT2* genotype, QFT-GIT and urine LAM, HRQOL for diagnosis and treatment monitoring of combined ATT and ARV in co-infected patients. Much work has been done on each of the subjects and using this information to evaluate whether these can be used together is important. The knowledge gap within specific groups in diagnosis of active and Latent TB and treatment monitoring of these. Diagnosis of smear-negative TB, sputum scarce groups and HIV-infected individuals is still wide and need investigation.

The next four chapters report on each of the 4 objectives of this thesis

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Chapter 3

N-acetyltransferase 2 genotypes among Zulu-Speaking South Africans and isoniazid and N-acetyl-isoniazid pharmacokinetics during anti-tuberculosis treatment

The possibility of individualised medicine has been investigated for the treatment of TB. NAT2 genotyping has the possibility of identifying patients at increased risk of adverse events to isoniazid. Knowing the prevalence of these polymorphisms in a population may help determine the best treatment dosing to reduce the experience of these adverse events.

Thesis Objective 1 – Assessment of polymorphisms in *NAT2* in participants receiving combined TB-HIV therapy.

Sub-objective 1.1- Determine genetic polymorphism frequency in the TB-HIV co-infected isiZulu-speaking black patients.

Sub-objective 1.2 - To evaluate the relationship of genetic polymorphisms to drug levels and inter-individual variability of INH.

Below, the manuscript responding to this thesis objective and published in Antimicrobial Agents and Chemotherapy journal is presented as published. The format has been changed to be consistent with the thesis format.

N-acetyltransferase 2 genotypes amongst Zulu Speaking South Africans and isoniazid / *N*-acetyl-isoniazid pharmacokinetics during anti-tuberculosis treatment

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Abstract

Background: Distribution of N-acetyltransferase 2 (*NAT2*) polymorphisms varies considerably among different ethnic groups. There is limited information on *NAT2* single nucleotide polymorphisms in the South African population. We investigated *NAT2* polymorphisms and their effect on isoniazid pharmacokinetics in Zulu black HIV-infected South Africans in Durban, South Africa.

Methods: Participants with culture-confirmed pulmonary tuberculosis (TB) were enrolled from two unrelated studies. Participants with culture-confirmed pulmonary tuberculosis (TB) were genotyped for *NAT2* polymorphisms 282C>T, 341T>C, 481C>T, 857G>A, 590G>A and 803A>G using Life Technologies pre-validated Taqman assays (Life Technologies, Paisley, UK). Participants underwent sampling for determination of plasma isoniazid and *N*-acetylisoniazid concentrations.

Results: Among the 120 patients, 63/120 (52.5%) were slow metabolisers (*NAT2**5/*5), 43/120 (35.8%) had intermediate (*NAT2*5/12*), and 12/120 (11.7%) had rapid genotype (*NAT2*4/*11*, *NAT2*11/12* and *NAT2*12/12*). *NAT2* alleles in this study *were *4, *5C, *5D, *5E, *5J, *5K, *5KA, *5T, *11A, *12A/12C* and **12M*. *NAT2*5* was the most frequent allele (70.4%) followed by *NAT2*12* (27.9%). Fifty-eight of 60 participants in study 1 had PK results. The median area under the concentration-time curve from 0 to infinity ($AUC0\text{-}\infty$) was 5.53 (interquartile range [IQR] 3.63 to 9.12 µg/ml/hr) and maximum concentration (C_{max}) was 1.47 µg/mL (IQR, 1.14 – 1.89 µg/ml). Thirty-four of 40 participants had both PK results and *NAT2* genotyping results. The median $AUC_{0\text{-}\infty}$ 10.76 µg/ml/hr (IQR, 8.24 – 28.96 µg/ml/hr) and C_{max} was 3.14 µg/ml (IQR, 2.39 – 4.34 µg/ml). Individual polymorphisms were not equally distributed, with some represented in small numbers. Genotype did not correlate with the phenotype, with those with rapid genotype showing higher $AUC_{0\text{-}\infty}$ than slow but not significant (p=0.43).

Conclusion: There was a high prevalence of slow acetylator genotypes, followed by intermediate then rapid acetylator genotypes. The poor concordance between genotype and phenotype suggests that other factors or genetic loci influence INH metabolism, and these warrant further investigation in this population.

KEYWORDS: acetylation, *N*-acetyltransferase, isoniazid, tuberculosis, HIV, pharmacokinetics, drug metabolism, pharmacogenetics

Introduction:

Tuberculosis (TB) remains a leading cause of global morbidity and mortality, with approximately 10 million cases and 1.5 million deaths in 2018 (1). South Africa is a high TB burden country with an estimated 301,000 cases in 2018. The so-called 'short-course' treatment regimen recommended in international guidelines; consisting of 6 months of rifampicin and isoniazid, supplemented by pyrazinamide and ethambutol in the first 2 months, has remained largely unchanged for several decades. Whilst this regimen can achieve high relapse-free cure rates, a range of host and mycobacterial factors can influence treatment outcomes. There is increasing evidence that inter-individual variability in the pharmacokinetics (PK) of drugs within this regimen lead to heterogeneity in clinical outcomes (2, 3).

Pharmacogenomics (PG) describe one cause of PK variability due to polymorphisms in drug metabolising enzymes and transporters. During TB treatment, isoniazid is the paradigmatic case. Isoniazid is acetylated to its major metabolite, N-acetyl-isoniazid (AcINH), by the action of hepatic N-acetyltransferase 2 (NAT2). AcINH is subsequently rapidly hydrolysed to acetyl-hydrazine, which is also acetylated, to diacetyl-hydrazine, by the action of NAT2 (4). Accumulated acetyl-hydrazine can be oxidised to form other, potentially hepatotoxic metabolites (4-6). Moreover, accumulated isoniazid can be metabolised by an alternative pathway where it is first hydrolysed to hydrazine, which has also been implicated in liver injury, before acetylation to acetyl-hydrazine, again by NAT2 (4, 7). Hence, the activity of NAT2 both dictates metabolism of isoniazid, and determines the availability of potentially hepatoxic hydrazine and acetyl-hydrazine metabolites. Within the 870-base pair NAT2 gene a number of low-activity single nucleotide polymorphisms (SNPs) have been characterised. The NAT2 genotype has been shown to determine the rate of acetylation by *NAT2* in several populations (8). Individuals homozygous for the wild-type alleles are characterised as 'rapid' acetylators (RAs), those homozygous for low-activity SNPs as 'slow' acetylators (SAs) and heterozygotes as 'intermediate' acetylators (IAs) (9-13). SAs have a higher incidence of side-effects, particularly drug-induced hepatitis, during TB therapy, presumably due to higher levels of hepatoxic metabolites (14-20). Amongst the first-line TB drugs isoniazid has the greatest early bactericidal activity (EBA) and isoniazid PK parameters have been associated with rates of cure, sterilisation and acquired drug resistance (3, 21-27). A link between rapid acetylation and increased risk of poor treatment outcomes has been reported (28, 29).

NAT2 genotype is known to differ amongst ethnic groups; with approximately 40-70% of Caucasians, Indians and African Americans characterised as SAs, versus only around 10% of Asian populations (30-

42). *NAT2* genotype is not well characterised in the communities where TB is most prevalent, particularly in sub-Saharan Africa. South Africa has several black ethnic groups and few have been studied (43-45). Bach et al. characterised 40% of a Zulu population as phenotypically slow acetylators but these findings have not been replicated, or informed by genotypic analysis (44). Moreover, South Africa has a high HIV prevalence and discordant relationships between *NAT2* genotype and isoniazid acetylator phenotype have been described amongst individuals living with HIV in other settings (46, 47).

We therefore characterised the relationship between *NAT2* genotype, isoniazid and *N-acteyl*-isoniazid PK and hepatotoxicity in a cohort of TB-HIV co-infected individuals in Durban, KwaZulu-Natal, South Africa.

Methods:

Participants, study treatment and sample collection

Participants from two unrelated PK studies were included (48, 49). Both studies recruited black, Zulu-speaking adults living with HIV from KwaZulu-Natal, South Africa, between March 2007 and April 2010. Study 1 was entitles "Bioavailability of the WHO Formulation Rifafour Containing Isoniazid, Rifampicin, Pyrazinamide, Ethambutol and the WHO recommended First Line Anti-Retroviral Drugs Zidovudine, Lamivudine, Efavirenz Administered to New TB Patients at Different Levels of Immunosuppression."(50). The results of this study have been previously reported (49). As shown in Table 9, for the purposes of this analysis, we used samples collected on day 1 of the study after an overnight fast, at pre-dose, 1, 2, 4, 5, 6, 8, and 12 hours post dose, with the samples being analysed for INH and AcINH for 60 participants with microbiologically proven pulmonary TB (by a positive sputum culture or smear) who received a standard first line TB regimen consisting of an FDC, as described above. The INH dose was 150 mg, 225 mg, 300 mg and 375 mg per day for participants with weight 30 -37 kg, 38– 54 kg, 55 – 70 kg, and 70 kg and above, respectively. Each participant had blood collected *NAT2* genotyping. In addition, genotyping was performed on a further 20 participants without TB who were initially recruited to this study (49).

Study 2, entitled "Pharmacokinetics of Rifabutin Combined with Antiretroviral Therapy in the Treatment of Tuberculosis Patients with HIV Infection in South Africa," was a randomised controlled trial of two different rifabutin doses co-administered with lopinavir/ritonavir-based antiretroviral therapy (51, 52). The participants initially received 6 weeks of standard intensive-phase treatment, followed by 2 weeks

with rifabutin 300mg daily replacing rifampicin. After 2 weeks of the continuation phase, during which participants received only isoniazid and rifabutin (both 300mg daily), PK sampling was carried out. Individuals were fasted overnight, and a standard hospital breakfast served 2 hours after drug ingestion. Sampling was conducted pre-dose and at 2, 3, 4, 5, 6, 8, and 12 and 24 hours after drug intake, with samples analysed for INH and AcINH for 40 participants. *NAT2* genotyping was performed on 40 participants with 34 participants having both PK sampling and genotyping.

All participants receiving anti-TB treatment in both studies were given pyridoxine for peripheral neuropathy prophylaxis and patients with CD4 counts below 200 cells/mm3 received cotrimoxazole. No participants were on antiretrovirals at the time of PK sampling. Both studies were approved by the South African Medicines Control Council (SAMCC), Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (Study 1- E294/05; Study 2- BFC011/07) and the South African Medical Research Council (SAMRC) ethics committee. Study one was also approved by the WHO Ethics Research Ethics Committee. Written informed consent was obtained from all participants.

NAT2 genotype procedures

Total Genomic DNA was isolated from whole blood using the QIAamp DNA mini kit (Qiagen, Crawly, UK) according to manufacturer's instructions. Participants were genotyped, using the DNA Engine Chromo4 system (Bio-Rad Laboratories, Hercules, CA) and Opticon Monitor v.3.1 software (Bio-Rad Laboratories), for 6 *NAT2* SNPs; 282C>T, 341T>C, 481C>T, 857G>A, 590G>A and 803A>G using Life Technologies pre-validated probe-based Taqman assays as per manufactures instructions (Life Technologies, Paisley, UK). Each participant sample was analysed in duplicate.

Haplotype assignment and acetylator genotype inference

Haplotype assignment from probe-based SNP data is poorly described in African populations. We elected to employ an unbiased PHASE analysis, which takes the dataset as a whole to assign the most likely haplotype for each individual, alongside a probability for this assignment (53, 54). Haplotype for each individual and acetylator genotype for each haplotype were defined as per the *NAT* Gene Nomenclature Committee (http://nat.mbg.duth.gr/Human%20NAT2%20alleles_2013.htm) (55). Individuals with two rapid alleles were defined as RAs, those with two slow alleles as SAs and those with one fast and one slow allele as IAs.

Isoniazid and *N-acetyl*-isoniazid PK and phenotype inference

Blood samples were collected and placed on ice immediately, before centrifugation within 60 minutes, immediate separation and storage of plasma at -70°C until analysis. Concentrations of isoniazid, AcINH and the 6-aminonicotinic acid internal control were quantified using validated high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). Sample preparation included a protein precipitation with acetonitrile and subsequent dilution with water. Analytes were chromatographically separated using a Waters Exterra C18, 3.5µm, 50mm x 2.1mm column and detected using the AB Sciex 5500 Q-Trap mass spectrometer. All analytes were analysed isocratically with an acetonitrile/water/0.1% formic acid mobile phase. isoniazid, AcINH and the internal standard were analysed at mass transitions of the precursor ions (m/z) 137.9, 180.1 and 138.7 to the product ions (m/z) 66.0, 78.6 and 50.9, respectively. Chromatographic data acquisition, peak integration and quantification of analytes was performed using Analyst® software version 1.5.2. We constructed time-concentration curves in the PK package in *R* for windows (version 3.5.1). We characterised the isoniazid and *N-acetyl*-isoniazid PK parameters maximum concentration (C_{max}), time to maximum concentration (T_{max}), area under the concentration curve from zero to infinity ($AUC_{0-\infty}$), apparent oral clearance (CL) and elimination half-life and compared C_{max} to published efficacy targets (56). $AUC_{0-\infty}$ was calculated using the trapezoid rule, apparent oral clearance estimated by dose / $AUC_{0-\infty}$ and elimination half-life was estimated by regression analysis of log concentrations of the terminal exponent of elimination. We analysed the ratio of log AcINH to log isoniazid at two and four hours to assess acetylation phenotype.

Sample processing and HPLC-MS was initially conducted in 2010 for Study 1. Samples remained in storage and were later moved to a new storage facility before they were shipped to a different laboratory for determination of isoniazid and AcINH concentrations as above (having previously only had isoniazid concentrations determined). To confirm the Integrity of these samples we compared the isoniazid $AUC_{0-\infty}$ of the current analysis with that previously reported on the same samples analysed in 2010.

Statistical methods

All data were entered in Epidata and transferred to either Stata (version 14) or R for windows (version 3.5.1) for statistical analysis. Demographic characteristics were presented as frequencies and percentages for categorical variables, and as means with standard deviations for continuous variables. Descriptive PK data was described as median and inter-quartile ranges. C_{max} and $AUC_{0-\infty}$ were log-transformed prior to

comparison between genotypes. PK parameters were compared, by genotype, using the Wilcoxon rank-sum test or Kruskal–Wallis test.

Hepatic adverse events

Hepatic adverse events were defined as elevated alanine transaminase (ALT) and aspartate transaminase (AST) levels, elevated alkaline phosphatase levels, and elevated total bilirubin levels, graded per the Division of AIDS, NIAID, NIH, toxicity table for grading the severity of HIV-positive adult adverse events.

Data availability. Data related to this study have been deposited at https://figshare.com/s/8b42c433e1edce625849.

Results:

Participant characteristics

One hundred and twenty-two individuals living with HIV participating in two PK studies were included in the study. Eighty participants in study 1 were included in the *NAT2* genotyping analysis and 60 in the PK analysis while 40 participants in study 2 were included in the PK analysis and 40 participants included in *NAT2* genotyping analysis (with 34 individuals having both PK and genotype data). Key characteristics are outlined in Table 4.

Table 4: Demographic characteristics

Characteristic	**Study 1 (n=80)**	**Study 2 (n=40)**	**Overall (n=120)**
Demographics			
Median (IQR) age (yr)	33 (18-48)	33.6 (24-53)	33.1 (18-53)
No. (%) male participants	36 (45)	24 (60.0)	60 (50)
No. (%) participants of Zulu ethnicity	80 (100)	40 (100%)	120 (100)
Mean (SD) weight (kg)	58.7 (11.9)	58.9 (9.7)	58.7 (11.2)
Mean (SD)BMI	23.0 (5.2)	23.1 (3.9)	23.1 (4.8)
No. (%) participants with BMI <18.5	13 (16.3)	2 (5.0)	15 (12.5)
Median CD4 (range)	210.5 (10-500)	128 (61 – 199)	161 (10-500)
CD4 < 200 (%)	40 (50)	40 (100)	80 (66.7)

Participants in study 1 included 60 with pulmonary TB and HIV co-infection; 40 with CD4 count >200 cells/mm^3 and, 20 with CD4 count <200 cells/mm^3 as well as 20 participants with HIV and without TB (who contributed only genotype data). All 40 participants in study 2 had TB and HIV co-infection, with a CD4 count of 200 cells/mm^3 or below. In the combined studies, 66.7% of participants had CD4 counts

<200 cells/mm^3 and 33.3% had CD4 count >200 cells/mm^3. The Median age was 33.1 years (IQR 18-53). Only 15 (12.5%) of patients had a BMI < 18.86 kg/m^2.

NAT2 genotype and deduced phenotype

One hundred and twenty participants (80 from study 1 and 40 from study 2) were genotyped. Haplotype assignment and deduced acetylator phenotype for each individual is shown in Table 6. Allele and haplotype frequencies and deduced phenotypes are outlined in Tables 11-13. We identified 12 different alleles in the population. The most common allelic group was *NAT2*5* (70.4%) followed by *NAT2*12* (27.9%). From the *NAT2*5* group *NAT2*5C* (21.3%), *NAT2*5J* (17.5%), *NAT2*5D* (14.6%) and *NAT2*5K* (10.4%) were the most common. The *NAT2*12* group was predominantly *NAT2*12C*. The deduced phenotype was 11.7% rapid, 35.8% intermediate and 52.5% slow acetylators (Table 9).

Table 5 Pharmacokinetic time points and dosing

Study	**Schedule of pharmacokinetic sampling (day of TB treatment)**	**Treatment**
Study 1	Days 1; with sampling pre-dose and at 1, 2, 4, 6, 8, 12 hours after the dose.	Four drug FDC formulation (EMB/ RMP/ INH/ PZA 275/150/75/400 mg, respectively) dosed daily by weight band
Study 2		Enrolment at week 6 and standard weight- band-based treatment with RMP, INH, PZA and EMB (as in study 1)
		At week 6 and 7: RMP replaced with RFB 300 mg daily
	Day 63 (after 2 weeks on continuation phase RBN-INH) with sampling pre-dose and 2, 3, 4, 5, 6, 8, 12 and 24 hours after the dose.	At week 8 and 9, RFB 300 mg and INH 300 mg

[a]PK= pharmacokinetics; RMP=rifampicin; PZA=pyrazinamide; EMB=ethambutol; FDC=fixed dose combination; RFB, rifabutin (Mycobutin, Pfizer).
[b]Participants weighing 30 to 37 kg received 2 tablets, those weighing 38 to 54 kg received 3 tablets, those weighing 55 to 70 kg received 4 tablets, and those weighing >70 kg received 5 tablets.

Isoniazid and *N-acetyl*-isoniazid PK

As above, to assess sample integrity for sample 1 we compared the AUC0-∞ of the current analysis with that previously reported on the same samples analysed in 2010. The median (IQR) AUC0-∞ was 5.53 (3.63 – 9.12), processed at University of Cape Town (UCT) in 2009 and 5.70 (3.85 – 7.94), processed at Africa Health Research Institute (AHRI) laboratory in 2014, suggesting that the integrity of the samples was maintained for isoniazid, but cannot be confirmed for N-acetyl-isoniazid.

Participants in study 1 showed rapid absorption, with a isoniazid median time to maximum concentration (T_{max}) of 1 hr (IQR, 1 -2h). Isoniazid exposure was variable among individuals, with the median maximum concentration C_{max} being 1.47 (IQR) (1.14 – 1.85) µg/ml and $AUC_{0-\infty}$ 5.53 µg.h/ml (3.63 – 9.12 µg.h/ml).

Median (IQR) elimination half-life was relatively slow at 2.27 h (1.69 – 3.56 h). We compared these isoniazid PK measures to published targets; 98.28% (57/58) failed to attain the minimum 2-hour plasma

Table 6. NAT2 diplotypes and genotypes and deduced phenotype in the study group.

Observed Diplotype†	n	Genotype	Phenotype
-20000	1	5D/5K	SLOW
000020	1	12A/12A	RAPID
001000	1	4/11A	RAPID
001020	6	12A/12C	RAPID
002010	2	11A/12C	RAPID
002020	4	12C/12C	RAPID
01-020	1	5C/12C	INTERMEDIATE
010010	1	5D/12A	INTERMEDIATE
010020	2	5C/12A	INTERMEDIATE
010110	2	5E/12A	INTERMEDIATE
011010	3	5D/12C	INTERMEDIATE
011020	15	5C/12C	INTERMEDIATE
011110	3	5E/12C	INTERMEDIATE
0200-0	1	5C/5D	SLOW
020000	1	5D/5D	SLOW
020010	10	5C/5D	SLOW
020020	3	5C/5C	SLOW
020100	3	5D/5E	SLOW
020110	1	5C/5E	SLOW
110010	1	5K/12A	INTERMEDIATE
110110	1	5K/12C	INTERMEDIATE
111010	5	5K/12C	INTERMEDIATE
111020	1	5T/12C	INTERMEDIATE
111110	6	5J/12C	INTERMEDIATE
120000	7	5D/5K	SLOW
120010	5	5C/5K	SLOW
120011	1	5C/5KA	SLOW
120020	1	5C/5T	SLOW
120100	7	5D/5J	SLOW
120110	8	5C/5J	SLOW
120200	1	5E/5J	SLOW
211020	1	5T/12M	INTERMEDIATE
211110	1	5J/12M	INTERMEDIATE
220001	1	5K/5KA	SLOW
220100	4	5J/5K	SLOW
220110	1	5J/5T	SLOW
2202-0	1	5J/5J	SLOW
220200	6	5J/5J	SLOW

†Observed diplotypes are shown as the number of mutations identified in each individual for each SNP. 0 = wild type, 1 = heterozygous, 2 = homozygous, - = blank. The SNP order is 282, 341, 481, 590, 803, 857.

concentration target of 3 µg/ml (56). PK parameters by genotype are shown in table 11A, unexpectedly median half-life was slowest, apparent oral clearance lowest and $AUC_{0-\infty}$ highest among genotypically rapid acetylators, with the reverse true for genotypically slow acetylators, although none of these differences was statistically significant. Similarly, there were no statistically significant differences by genotype for N-acetyl-isoniazid C_{max}, elimination half-life or $AUC_{0-\infty}$. Median isoniazid and AcINH time-concentration curves are given in *Figure* 2 (Left).

Table 7. Frequency of NAT2 alleles in the study group.

Polymorphism	No. of participants	% of participants
NAT2*4	1	0.4
NAT2*5	169	70.4
NAT2*11	3	1.3
NAT2*12	67	27.9
Total	240	100

Table 8. Frequency of NAT2 haplotypes.

Haplotype	No. of participants	% of participants
NAT2*4	1	0.4
NAT2*5C	51	21.3
NAT2*5D	35	14.6
NAT2*5E	10	4.2
NAT2*5J	42	17.5
NAT2*5K	25	10.4
NAT2*5KA	2	0.8
NAT2*5T	4	1.7
NAT2*11A	3	1.3
NAT2*12A	14	5.8
NAT2*12C	51	21.2
NAT2*12M	2	0.8
Total	240	100

Table 9: Frequency distribution of NAT2 genotypes and deduced phenotype in the study group.

Genotype	n	%	Acetylator status
NAT2*4/*11	1	0.8	
NAT2*12/*12	11	9.2	RAPID
Nat2*11/*12	2	1.7	
NAT2*5/*12	43	35.8	INTERMEDIATE
NAT2*5/*5	63	52.5	SLOW
Total	120	100	

Absorption was rapid in study 2, with a median INH Tmax being 2 hrs. INH exposure was also variable amongst individuals with median (IQR) Cmax 3.14 µg/ml (2.39 – 4.34 µg/ml) and the median AUC0-∞ 10.76 µg.hr/ml (8.24 – 28.96 µg/ml). The median elimination half-life was 2.62h (2.26 – 4.07h). Again, we compared these INH PK measures to published PK targets; 47.5% (19/40) failed to attain the minimum 2-h plasma concentration target of 3 µg/ml.

Table 10: Overall isoniazid and N-acetyl-isoniazid PK

	Study 1		Study 2	
	Isoniazid	*N*-acetylisoniazid	Isoniazid	N-acetylisoniazid
$AUC_{0-\infty}$ (µg/mL/hr)	5.53 (3.63 – 9.12)	5.49 (3.18 – 9.26)	10.76 (8.24 – 28.96)	27.67 (23.20 -34.67)
C_{max} (µg/mL)	1.47 (1.14 – 1.89)	0.90 (0.46 – 1.398)	3.14 (2.39 – 4.34)	2.91 (1.73 – 3.70)
T_{max} (hr)	1 (1 – 2)	4 (2 – 6)	2 (2 - 2)	3 (3 – 4)
CL/F (L/hr)	47.64 (35.36 – 74.11)	NA	27.34 (10.83 – 32.00)	NA
$t_{1/2}$ (hr)	4.61 (3.64 – 8.32)	10.64 (6.62 – 17.07)	6.02 (5.37 – 8.66)	8.03 (6.18 – 12.86)

All values medians (inter-quartile ranges)
$AUC_{0-\infty}$ = Area under the time – concentration curve
C_{max} = Maximum concentration
CL/F = Clearance
$t_{1/2}$ = Elimination half life
NA = not applicable

PK parameters by genotype are shown in Table 11 B. For both isoniazid and AcINH and across the PK parameters; C_{max}, $AUC_{0-\infty}$ and elimination half-life, variability (both range and IQR) was increased amongst those genotyped as SAs. Again, however, there were no statistically significant differences between these PK parameters by genotype. Median isoniazid and AcINH time-concentration curves are given in Figure 2.

Table 11A: Study 1 PK parameters by genotype

	Isoniazid			*N*-acetyl-Isoniazid		
	Slow	Intermediate	Rapid	Slow	Intermediate	Rapid
$AUC_{0-\infty}$ (µg/mL/hr)	5.34 (3.44 – 7.93)	6.04 (4.27 – 7.53)	7.56 (5.99 -9.60)	5.71 (4.19 – 11.01)	7.34 (3.15 – 10.9)	2.81 (0.55 – 5.06)
C_{max} (µg/mL)	1.47 (0.97 – 1.89)	1.54 (1.25 – 1.76)	1.42 (1.20 -2.05)	0.94 (0.63 – 1.68)	1.07 (0.49 – 1.70)	0.38 (0.90 – 0.90)
T_{max} (hr)	1 (1 -2)	1 (1 – 2)	2 (2 – 2)	4 (2 – 4)	4 (4 – 7)	6 (4 -6)
CL/F (L/hr)	57.05 (37.84 – 103.56)	43.53 (32.05 – 64.33)	37.75 (31.27 – 47.92)	NA	NA	NA
$t_{1/2}$ (hr)	4.67 (3.64 – 8.32)	4.00 (3.35 – 5.19)	8.56 (5.69 – 14.44)	9.42 (5.75 – 17.07)	6.55 (6.68 – 10.93)	14.78 (10.65 – 22.41)

All values medians (inter-quartile ranges)
$AUC_{0-\infty}$ = Area under the time – concentration curve
C_{max} = Maximum concentration
CL/F = Clearance
$t_{1/2}$ = Elimination half life
NA = not applicable

For both studies we calculated the log AcINH concentration/log isoniazid concentration ratio, as a measure of acetylation, at 2 and 4 hours postdose and analysed this ratio by genotype (Figure 3 and 4). In both studies we saw no statistically significant difference in ratios between genotypes at either 2 or 4 hours. In study 2 we again saw increased variability in this metric amongst those genotyped as SAs.

Table 11B: Study 2 PK parameters by genotype

	Isoniazid			***N*-acetyl-Isoniazid**		
	Slow	**Intermediate**	**Rapid**	**Slow**	**Intermediate**	**Rapid**
$AUC_{0-\infty}$ (µg/mL/hr)	10.76 (9.73 -31.21)	9.09 (7.3 -18.75)	26.99	26.04 (22.99 -32.76)	6.28 (5.25 -10.01)	28.53
C_{max} (µg/mL)	3.47 (2.49 - 4.49)	2.96 (2.33 - 4.02)	3.94	2.85 (1.52 - 3.68)	3.28 (2.53 - 4.01)	1.91
T_{max} (hr)	2 (2 – 2)	2 (2 – 2)	2	3 (3-4)	3 (3 – 3)	4
CL/F (L/hr)	27.87 (9.66 -30.83)	33.33 (16.01 - 41.17)	11.12	NA	NA	NA
$t_{1/2}$ (hr)	4.61 (3.9 -5.34)	4.46 (3.9 - 7.88)	8.28	5.81 (4.9 - 7.25)	6.28 (5.25 -10.01)	10.97

All values medians (inter-quartile ranges)
$AUC_{0-\infty}$ = Area under the time – concentration curve
C_{max} = Maximum concentration
CL/F = Clearance
$t_{1/2}$ = Elimination half life
NA = not applicable

Hepatic adverse events

There were no grade 3 and 4 hepatic adverse events in study 1 and only 1 grade 4 hepatic event was reported from the only participant with rapid genotype in study 2. Although there were more hepatic

adverse events among the slow genotype participants, the difference was not statistically significant between genotypes; p=0.203 in study 1, and 0.276 in study 2.

Table 12: Participants with any hepatic adverse events

	Study 1				Study 2			
AE Grade	Rapid N(%)	Intermediate N(%)	Slow N(%)	**Total N(%)**	Rapid N(%)	Intermediate N(%)	Slow N(%)	**Total N(%)**
1	7	9	25	**41**	0	5	10	**15**
2	2	0	0	**2**	0	1	1	**2**
3	0	0	0	**0**	0	2	1	**3**
4	0	0	0	**0**	1	0	0	**1**
Total	**9 (20.9)**	**9 (20.9)**	**25 (61)**	**43 (100)**	**1(4.8)**	**8(30.1)**	**12(57.1)**	**21(100)**

Discussion:

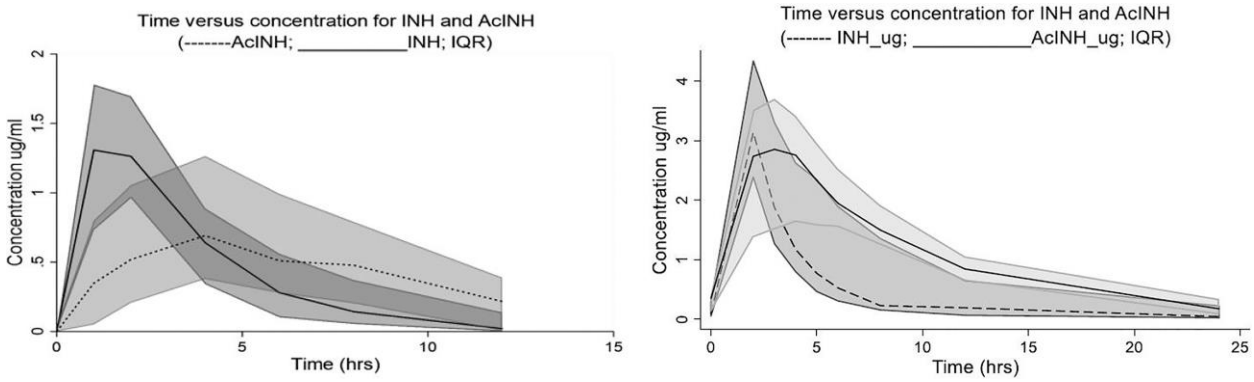
We investigated the NAT2 genotype and the PKs of isoniazid and AcINH in black Zulu South Africans living with HIV from Durban and surrounding areas. We found that most individuals were of SA (52.5%) or IA (35.8%) genotype, with only a small number of RA genotype (11.7%). The proportions of the deduced acetylator phenotypes in our population was broadly similar to other African and Caucasian populations (36, 43, 57, 58) but differ from those previously reported from within other black ethnic groups within Southern Africa. For example, Werely found that IA genotypes dominated in the Xhosa cohort, with SAs only 30% (45). Our results were comparable to a recent study by Naidoo et al. in patients from the same geographic area who reported 34% SA, 43% IA and 18% RA (59).

There was a high prevalence of the NAT2*5 allelic group, accounting for the slow acetylator genotype, in our population. In well studied Caucasian and Asian populations, four variants; *NAT2*4* (wild type, rapid acetylators), *NAT2*5B*, *NAT2*6A*, and *NAT2*7B* (all slow acetylators), account for most *NAT2* alleles. In Asian populations there are generally a higher proportion of wild type *NAT2*4* alleles and few *NAT2*5B* alleles, and this difference largely accounts for the much lower prevalence of RAs in non-Asian populations. Consistent with other studies in Sub-Saharan African populations, the wild-type *NAT*4* allele was far less prevalent and variant alleles were far more diverse in our study. In our population, the *NAT2*5B* allele was relatively rare in comparison to its occurrence in two studies in the black population from Western Cape and North West Province in South Africa (45, 60).

However, in contrast to these South African populations, there were a diversity of other *NAT2*5* alleles, including a much higher prevalence of the rare *NAT2*5J* allele (17.5%) and the poorly characterised

*NAT2*5K* allele (10.4%). The *NAT2*6A* and *NAT2*7B* alleles, common in Caucasian and Asian populations, were not seen in our cohort. In Caucasian and Asian populations, rapid acetylator *NAT2*12* alleles are rarely seen, whereas in populations in sub-Saharan Africa the *NAT2*12A* allele is reported at much higher frequencies (35). In our study, the *NAT2*12A* allele did indeed comprise 5.8% of alleles seen, but we saw a much higher frequency of the *NAT2*12C* allele (21.2%), in contrast to other Southern African cohorts (10, 45, 60, 61).

Figure 2 (Left) Study 1 median INH and AcINH concentrations over time for INH and AcINH for 58 patients. (Right) Study 2 median INH and AcINH concentrations over time for INH and AcINH for 34 patients. The shaded areas indicate the IQR.



The isoniazid C_{max} and AUC0-∞ demonstrated considerable variability between individuals in both studies, and almost all participants in study 1 and almost half of the participants in study 2 had a C_{max} below the lower limit of the target range (56). Low isoniazid concentrations during TB treatment are concerning because it is postulated they may lead to poorer treatment outcomes, or the generation of isoniazid resistance, the likely first step in the evolution of multi-drug resistant TB (MDR TB). However, the evidence for either of these concerns is mixed and in this setting the prevalence of INH mono-resistance is relatively low.

There was a marked difference in PK measures between the two studies analysed, with Study 1 having much lower measures than Study 2. There are several reasons that could have contributed to this difference. The difference in isoniazid dosing could explain the lower PK measures, where Study 1 used the FDC dosing as per WHO recommended weight bands, leading to almost half the participants receiving doses <300 mg, as previously reported (49). All participants in Study 2 received 300 mg doses of isoniazid irrespective of weight. Although the samples of Study 1 did not appear to deteriorate during the 5 years between first analysis and subsequent analyses for this study, differences in processing and storage

between the studies cannot be excluded. Figure 4 shows the INH and AcINH at different time points. Based on those findings, the phenotype of the study participants is generally more intermediate/rapid than what the predominant slow genotype suggests, which is in contrast to other studies reporting HIV-infected patients have a tendency towards slow acetylator phenotype (62).

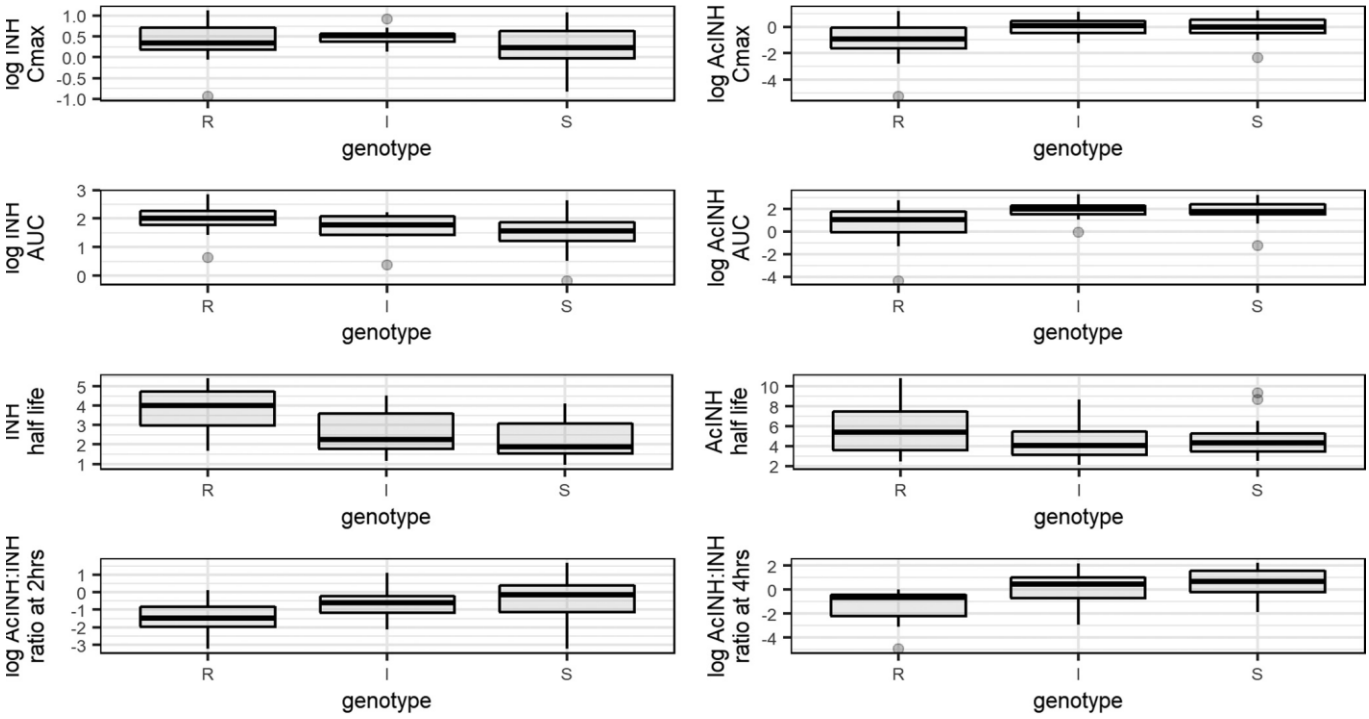


Figure 3 Box plots for study 1:
Representing median (solid line), interquartile range (box) and range (whiskers) for the pharmacokinetic parameters; log maximum concentration (Cmax), log area under the time-concentration curve (AUC0-∞), and half-life for isoniazid (INH) and N-acetyl-isoniazid (AcINH) stratified by acetylator status and logAcINH concentration to logINH concentration ratio at 2 and 4 hours stratified by acetylator genotype, R,I, and S, rapid, intermediate and slow acetylator genotypes, respectively.

We identified no statistically significant difference by *NAT2* genotype in a variety of PK measures, hence, in this cohort we found poor correlation between *NAT2* genotype and phenotypic acetylation of isoniazid. Previous studies in other populations have shown a good correlation between *NAT2* genotype and isoniazid PK, suggesting that *NAT2* genotyping could be used as a parsimonious way to risk stratify patients and personalise dosing of isoniazid in an attempt to maximise efficacy whilst minimising toxicity. There are significant practical difficulties to implementing these approaches in this setting, but our data suggest that in this population *NAT2* genotyping will not be helpful in guiding TB therapy. A lack of concordance between genotypic and phenotypic measures of INH acetylation has been reported previously in HIV-positive cohorts (63, 64). It is likely that in this cohort, as in others, that other non-genetic factors are more or equally important than *NAT2* genotype. Jones et al found that infection with HIV or stage of

HIV infection may alter Phase I and II drug-metabolising enzyme (DME) activity in their study on 17 HIV-infected participants at different levels of immunosuppression (65). They found that HIV infection was related to an increase in variability of these DMEs. Whilst additional pathways, aside from *NAT2* genotype, have been implicated in hepatotoxicity of isoniazid-containing TB treatment regimens, it is not clear that these pathways alter isoniazid PK and thus could account for the lack of genotypic and phenotypic concordance in this study.

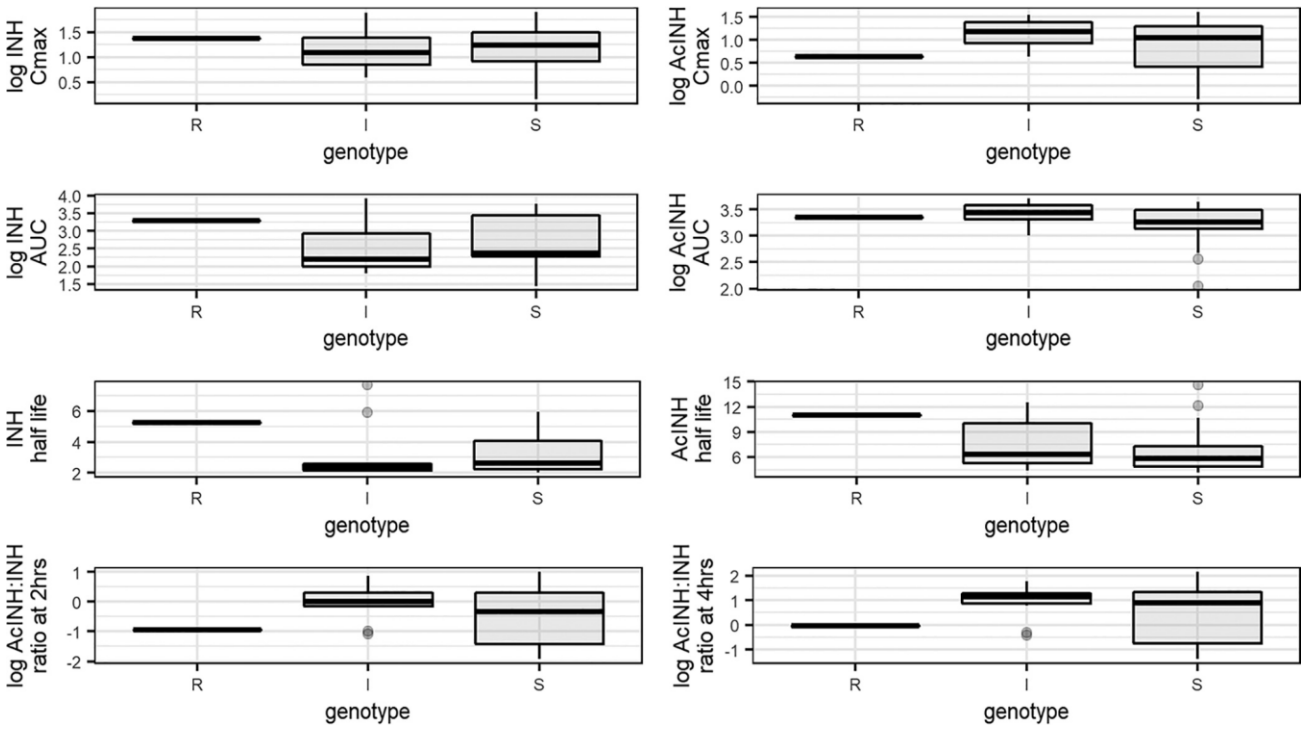


Figure 4 Box plots for study 2:

Representing median (solid line), interquartile range (box) and range (whiskers) for the pharmacokinetic parameters; log maximum concentration (C_{max}), log area under the time-concentration curve ($AUC_{0-\infty}$), and half-life for isoniazid (INH) and N-acetyl-isoniazid (AcINH) stratified by acetylator status and logAcINH concentration to logINH concentration ratio at 2 and 4 hours stratified by acetylator genotype, R,I, and S, rapid, intermediate and slow acetylator genotypes, respectively.

Although there were more hepatic adverse events among the SA, there was no statistical association between genotype and hepatotoxicity in the two studies, with only 1 patient who was a RA having a grade 4 hepatic adverse event and 2 others who were IA having grade 3 hepatic adverse events.

In our study, the participants received pyridoxine and cotrimoxazole with the ATT in study 2, but not in study 1 as we used the samples collected on day 1 for this analysis when only ATT was given. As both INH and sulfamethoxazole are inhibitors of CYP2C9, this could be one of the reasons for the variations noted. INH also inhibits CYP3A4, which is induced by rifampicin, but this interaction has not proven

significant except when it relates to hepatotoxicity (66, 67). That the combination of INH and rifampicin leads to an increased risk of hepatotoxicity, has been reported in other studies. In our study 2, isoniazid was given with Rifabutin which is a less potent hepatic enzyme inducer, which therefore should have less interaction with INH (68). Considering the limited effect on hepatotoxicity, the effect of CYP2E1 was not evident in our study. We cannot confirm or exclude the effect of these CYP450 enzymes on INH metabolism in these participants.

In our study, samples were stored at -80^0 Celsius and loss of compound due to storage would have been minimal (69), although studies have not reported on plasma samples stored longer than 5 weeks, nor have they reported on sample integrity for the metabolite, AcINH.

Conclusion

Amongst black Zulu TB-HIV-co-infected South African patients, most had slow or intermediate acetylator *NAT2* genotype. There was a diversity of specific *NAT2* alleles, with the pattern differing from previously studied cohorts in other settings. Despite the rarity of rapid acetylator genotypes, INH PK were variable and a substantial proportion of individuals failed to attain minimum efficacy targets. Importantly, *NAT2* genotype did not explain PK variability in this cohort or the low C_{max}, which suggests that other factors could be influencing isoniazid bioavailability and metabolism, and which require further elucidation.

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Chapter 4

Impact of tuberculosis treatment and antiretroviral therapy on serial RD-1-specific quantitative T-cell readouts (QuantiFERON®-TB-Gold-in-Tube), and relationship to treatment-related outcomes and bacterial burden

Treatment monitoring of TB is commonly done using sputum microscopy. This is dependent on patients' ability to produce sputum. In TB and HIV co-infected patients often cannot produce sputum. Studies on the usefulness of IGRAs as treatment monitoring tools have been conducted in TB patients and very few were conducted in co-infected patients.

Objective 2 - To determine the kinetics of mycobacterial cellular immune responses in patients treated with HAART and TB drugs using an INF-γ release assay.

The manuscript below reports on the kinetics of INF-γ using QuantiFERON®-TB-Gold-in-Tube in TB and HIV co-infected patients receiving combined treatment. All patients recruited were ART naïve before the study. This was a longitudinal study with a twelve month follow up; enrolment started in 2007 and follow up ended in 2010. The samples for QFT-GIT were processed without first being frozen. The delay in publication of the manuscript was due to various factors, including staff migration.

Impact of tuberculosis treatment and antiretroviral therapy on serial RD-1-specific quantitative T-cell readouts (QFT-GIT), and relationship to treatment-related outcomes and bacterial burden

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ABSTRACT

Background: The impact on anti-TB treatment with and without antiretroviral therapy (ART) on standardised interferon-γ release assay (IGRA) readouts has been inadequately studied in high burden countries.

Methods: The QuantiFERON®-TB-Gold-in-Tube (QFT-GIT) test was used to evaluate (IFN-γ) responses longitudinally (0, 3, 6 and 12 months post initiation of tuberculosis (TB)-HIV co-treatment or ART alone) in 82 HIV-infected patients.

Results: Of the 65 evaluable participants 30 were co-infected on ART, 17 co-infected but not on ART, and 18 were HIV-infected alone but on ART. In HIV-infected and HIV-TB-infected patients on ART IFN-γ responses increased, whilst they decreased in those not on ART. However, baseline, month 3, and month 6 IFN-γ responses, irrespective of ART, did not differ in TB-HIV co-infected patients who culture-converted compared to those who did not (1.25 vs. 1.05, p=0.5 at baseline; 3.76 vs. 1.15, p=0.2 for month 3; and 0.06 vs. 0.7, p= 0.3 for month 6, respectively). IFN-γ levels did not correlate with the magnitude of sputum bacillary load, smear status, or liquid culture time-to-positivity.

Conclusion: As IGRAs do not correlate with 2 or 6-month culture conversion and markers of bacillary burden, they are unlikely to be useful for the prognostication of treatment outcome in co-infected patients.

KEYWORDS: tuberculosis, HIV, antiretrovirals, interferon-γ release assay, QuantiFERON® TB Gold-in-Tube

Introduction:

There is a desperate need for new tuberculosis (TB) diagnostics and biomarkers to assist in new drug and vaccine development. Presently phase III clinical trials are conducted over a period of 30 to 60 months because it must be shown that a drug or drug combination prevents or reduces treatment failure and relapse (which requires a 24 month follow-up period) (1). Recently it has been suggested that a shorter follow may be possible, as studies have shown that if patients were to relapse, they would do so within 12 months of completion of anti-TB treatment (ATT) (2, 3). Similarly, vaccine studies suffer from a lack of biomarkers that can predict a favorable response. Monitoring TB treatment response using sputum smear and culture poses problems as human immunodeficiency virus (HIV)-infected patients are frequently smear-negative (4) and these tests themselves are suboptimal predictors of outcome (5). More recently interferon gamma release assays (IGRAs) have become available and have been investigated for use as diagnostics and biomarkers of TB treatment response and prognosis (6-27).

Some studies have reported that IGRAs may be useful in monitoring TB treatment response (23, 28), while others have reported that most patients remain IGRA-positive after completion of ATT, in spite of sputum smear and culture conversion (12, 29). These studies have examined the utility of measuring longitudinal IFN-γ responses during the course of TB treatment. They have mostly been conducted in pre-dominantly HIV-uninfected individuals (12, 21, 30-32) and mainly in countries with low TB and HIV burdens (11, 33-35). Only two studies have used the commercially available standardised QuantiFERON®-TB Gold in Tube (QFT-GIT) assay in a setting with a high burden of TB and HIV (24, 36). However, to our knowledge, no study has hitherto reported effects of ART and/ or ATT on quantitative IFN-$_{\gamma}$ responses in HIV-TB co-infected patients. There are also hardly any data about the effect of ART alone on such responses. A further limitation is that several published studies, even from Africa, have used non-standardised in-house IGRAs in an enzyme-linked immunosorbent assay format (16, 37). Although there have been reports that IGRAs may correlate with bacterial load in low burden settings (38, 39), this was not the case in the only published report in patients from a high burden setting (40). There is therefore a need for more data from high burden settings, such as South Africa, where TB prevalence was estimated to be between 0.4 million and 0.6 million cases in 2012 (41).

To address the deficiency in our knowledge about how T-cell responses change during the course of therapy we investigated the usefulness of QFT-GIT in monitoring TB treatment responses over a one year period in HIV-infected patients receiving standard ATT with or without ART at primary care clinics in

Durban, South Africa. The inclusion of patients on ART makes this study important in addressing this gap in the literature.

Methods

Participants

As part of a parent pharmacokinetic study, we recruited HIV-infected patients aged 18 years through 65 years between March 2007 and April 2008 after written informed consent was obtained. This included 62 newly diagnosed TB patients with smear-positive disease who fall into three subgroups: (i) with a CD4 count <200 cells/mm^3 and initiated on ART as per national guidelines (n=20; henceforth referred to as TB-ART-<200); (ii) with a CD4 count >200 cells/mm^3 and initiated on ART (n=21; henceforth referred to as TB-ART->200); and (iii) with a CD4 T-cell count >200 cells/mm^3, not initiated on ART (n=21; henceforth referred to as TB-no-ART->200). We also enrolled patients without active TB disease (culture-negative) with CD4 T-cell count < 200 cells/mm^3 and who were initiated on ART (n=20; henceforth referred to as non-TB-ART-<200). The different patient groups are shown in Figure 7. Inclusion and exclusion criteria as reported previously (42). The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (E294/05) and was conducted according to ICH and South African Good Clinical Practice. All subjects signed informed consent. This study is registered with controlled-trials.com, ISRCTN77861053.

Recruitment and specimen collection

TB patients were enrolled if they were smear- and culture-positive, as well as smear- and culture-negative non-TB patients. Blood samples were drawn for the QFT-GIT (Cellestis Ltd., Carnegie, Australia) assay at 0, 3, 6 and 12 months from all enrolled participants. Sputum specimens were collected for smear and culture at month 0, 2, 5 and 6 during TB treatment, and at every follow up visit (month 9, 12, 15, 18 and 24) if they showed signs and symptoms of TB.

Laboratory procedures

Sputum samples were processed using NALC/NaOH. Smear microscopy using both auramine and Ziehl-Neelsen staining (43), and culture using Middlebrook 7H11 selective agar (Difco) and Mycobacteria Growth Indicator Tube (MGIT) liquid medium (Becton Dickenson Biosciences Division, Sparks, Maryland, USA). Time to obtain a positive result was recorded as the growth was observed, or negative if at 42 days there was no growth. Positive cultures were confirmed for *M. tuberculosis* complex using

niacin and nitrate biochemical testing and tested for susceptibility to antimicrobial agents using the proportion method on Middlebrook 7H10 agar (Difco).

The QFT-GIT assay was performed according to manufacturer's instructions (44). QFT-GIT blood collection tubes, which include a Nil Control tube, TB Antigen tube and an optional Mitogen tube were incubated at 37°C as soon as possible and within 16 hours of collection. Following a 16 – 24 hour incubation period, the tubes were centrifuged, the plasma removed and the amount of interferon gamma (IFN-γ) measured by enzyme linked immunosorbant assay (ELISA). The results were interpreted using software supplied by the manufacturer and was considered positive if the response to the specific antigens was ≥0.35 IU/ml; negative if the response to the specific antigen was <0.35 IU/ml; the IFN-γ level of the positive control was ≥0.5 IU/ml and indeterminate if the value of both antigen stimulated samples were <0.35 IU/ml and the positive control was <0.5 IU/ml.

Data management and statistical analysis

The sample size was that of convenience. All data were entered using double data entry into an Epidata database and transferred to Stata version 11 (StataCorp. 2009, College Station, TX) for statistical analysis. TB and non-TB patient characteristics were compared using chi square tests for categorical and t-test for continuous variables. Median quantitative responses of QFT-GIT were compared across subgroups at baseline using the Wilcoxon Rank sum test. Differences between baseline and 3 and 6 months were tested using a paired t-test and differences between the subgroups tested using unpaired t-tests. There were 17 participants that withdrew or were withdrawn from the study for different reasons. The TB-ART CD4 > 200 group (n=10) and the TB-ART CD4 < 200 group did not differ in their QFT responses at baseline and follow up and were thus combined.

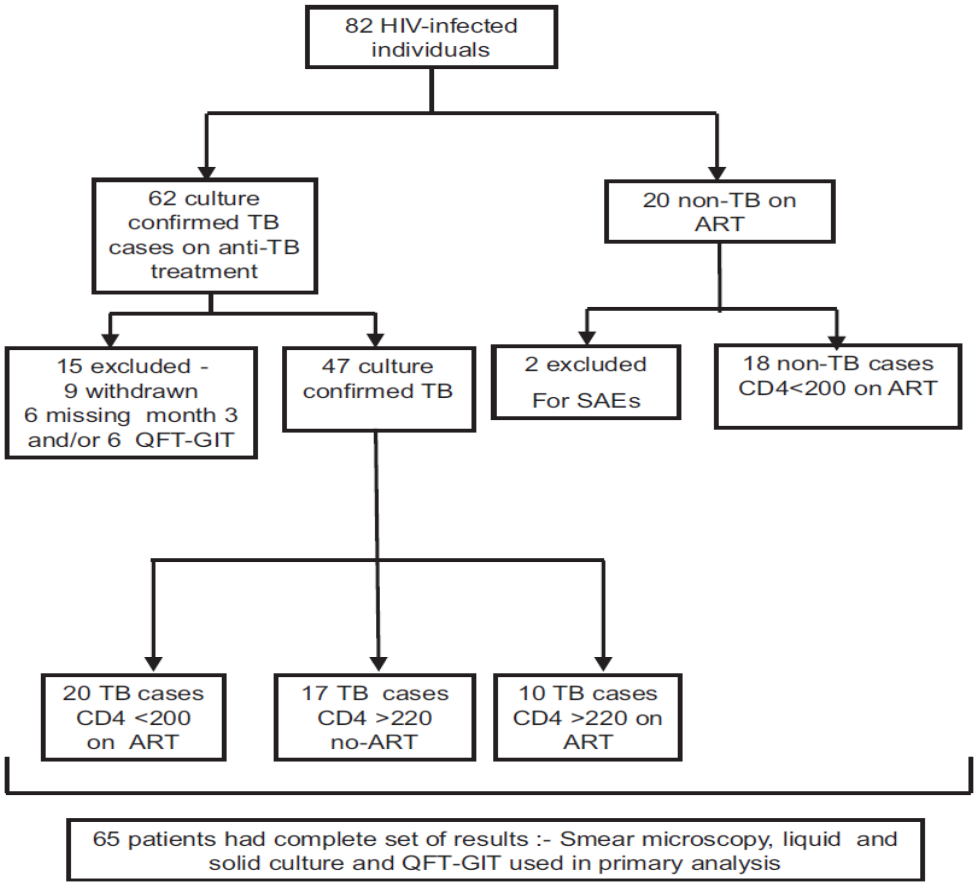

Figure 5 Study participants flow chart

Of the 82 participants recruited, 11 participants [9 in the TB arms (8 in TB-ART and 1 in the TB-no-ART group) and 2 in the non TB-ART group)] either withdrew consent or suffered a serious adverse event and had to be withdrawn from the study after recruitment. Six were excluded for incomplete tests; either missing month 3, 6 or both QFT-GIT results.

CD4 unit = cells/mm^3, TB = tuberculosis, QFT-GIT = QuantiFERON®-TB Gold In-tube, ART = antiretroviral therapy

Results:

Participant characteristics

One thousand and thirty-four patients were pre-screened and 82 were enrolled into the study. Eighteen were excluded because of World Health Organization (WHO) clinical stage 4 disease. Only 20 TB-HIV patients and 20 HIV-only patients with a CD4 count below 200 cells/mm3 were recruited and the rest had to have CD4 count above 200 cells/mm^3. Most exclusions were as a result of failing to fulfill the CD4 count criterion. Of the 82 participants, 65 had at least three of the 4 time point-specific QFT-GIT results and were therefore analysed. There were no significant differences in the demographic and baseline characteristics between the three groups (Table 13) in age, sex, weight, height, body mass index (BMI) and haemoglobin. There was no difference in the mean viral load between the groups at baseline (p = 0.35).

Table 13: Demographics of patients included in the analysis

Demographic or clinical characteristics	**TB-ART**			**TB-no-ART**	**no-TB-ART**
	CD4 < 200 cells/mm^3 **(n = 20)**	**CD4 > 200** cells/mm^3 **(n = 10)**	**P-value**	**CD4 > 200** cells/mm^3 **(n = 17)**	**CD4 < 200** cells/mm^3 **(n = 18)**
Age Mean years (SD)	36.2 (7.04)	29.4 (7.85)	0.02	29.6 (5.40)	35.5 (6.73)
Sex, n (%)					
Male	7 (35)	4 (40)	0.8	11 (65)	8 (44)
Female	13 (65)	6 (60)		6 (35)	10(56)
Weight (kg), mean (SD)	56.1 (12.69)	54.5 (7.12)	0.8	57 (11.94)	62.8 (11.58)
Height (m), mean (SD)	1.6 (0.09)	1.6 (0.11)	0.9	1.6 (0.11)	1.6 (0.11)
BMI (kg/m^2), mean (SD)	22.0 (4.52)	21.7 (3.73)	0.9	22.4 (5.34)	25.3 (6.13)
BMI category, n(%)					
Underweight	4 (20)	3 (30)		2 (0.12)	2 (0.11)
Healthy	12 (60)	6 (60)		13 (0.76)	8 (0.44)
Overweight	3 (15)	1 (10)	0.9	0 (0)	3 (0.17)
Obese	1 (5)	0 (0)		2 (0.12)	5 (0.28)
Haemoglobin, g/l), mean (SD)	10.1 (1.37)	11.2 (2.12)	0.3	11.1 (1.52)	11.0 (1.66)
CD4 count*, Mean (SD)	113.6 (54.39)	304 (75.05)	<0.001	331.9 (78.76)	120.3 (51.66)
Viral Load (log_{10})	5.2 (1.04)	4.9 (0.83)	0.2	4.7 (1.08)	

Eight participants in arm 1 withdrew, three of whom completed the pharmacokinetic study (PK), two were not ready to start ART, two refused to provide a blood sample and one had a grade 4 amylase. Three patients in arm 3 were withdrawn, one for grade 4 anaemia, one refused to provide a blood sample and the last had a repeat CD4 count above the enrolment threshold. In arm 2, four participants were withdrawn: one refused to provide a blood sample and three completed PK but had missing QFT-GIT results on two or three visits. Two participants in arm 6 died before month 6.

Baseline QFT-GIT results

From the 82 participants enrolled, only four had indeterminate results and only 65 had at least 3 sets of results; of these, only one had an indeterminate result which was regarded as negative. There were no significant differences in mean baseline IFN-γ levels between the TB-ART and TB-no-ART groups (2.12 vs. 2.95 IU/ml, p=0.2) (Figure 5). There was a significant difference between the non-TB-ART and the TB-ART groups (0.95 vs. 2.12 IU/ml, p<0.001) and non-TB-ART vs. the TB-no-ART group (0.95 vs. 2.95 IU/ml, p<0.001). IFN-γ levels were significantly higher in the TB groups (TB-ART and TB-no-ART) when compared to the non-TB-ART group, (1.15 vs. 0.24 IU/ml; p<0.001). The correlation between IFN-γ levels and CD4 count for all groups (TB and non-TB) was positive, r_s=0.26, p=0.02. The median baseline IFN-γ levels also differed significantly when stratified by CD4 count group (CD4 <200: 0.7 (IQR: 0.05–2.0) vs. CD4 > 200: 2.0 (IQR: 0.7–4.4), p=0.02. However, among the TB patients alone, the spearman's

correlation between IFN-γ and CD4 count was still positive, r_s=0.15 but no longer reaches statistical significance, p=0.30. The subsequent comparison of median QFT responses stratified by CD4 count in the TB group only (CD4< 200: 1.1 (IQR: 0.7 – 2.5) IU/ml vs. > 200: 2 (IQR: 0.7-4.4) IU/ml, p = 0.40) was not significant. Because there was no difference in the baseline IFN-γ in the two TB-ART groups, the results were pooled.

IFN-γ levels and bacillary load correlates at diagnosis

There was no correlation between IFN-γ levels and time to positivity [rs=0.09, p=0.90 (Figure 7 Panel A)]. The association between sputum bacillary load as measured by smear grade and IFN-γ levels was not significant (Figure 7, Panel B). IFN-γ levels did not significantly correlate with increasing smear grade (p=0.45).

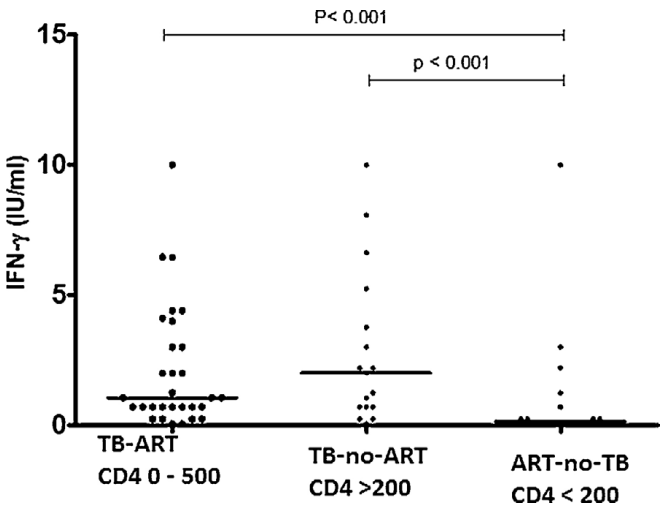

Figure 6. Baseline interferon gamma (IFN-γ) levels

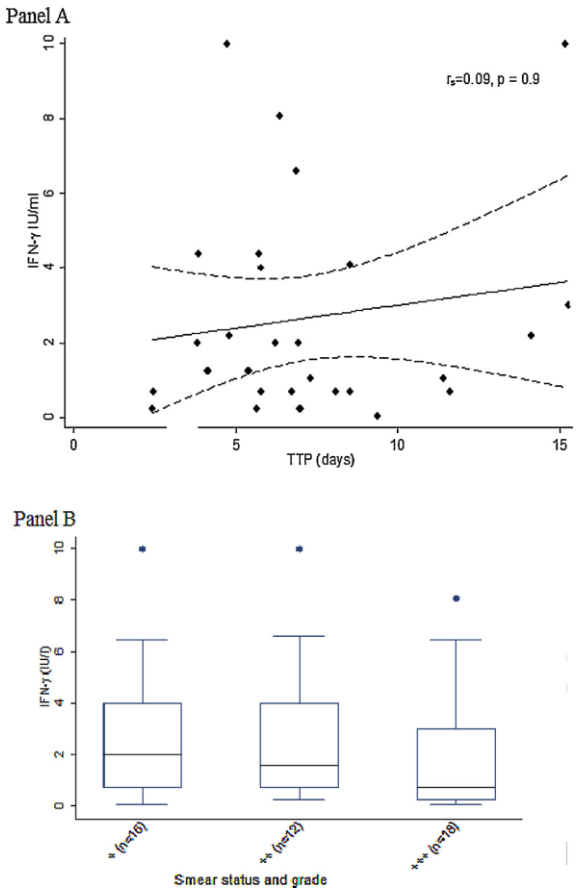

Figure 7. Panel A: Relationship between interferon gamma (IFN- γ) responses and bacillary load and time to positivity at baseline. Panel B: There were no scanty results. +, positive; ++, 2+ positive and +++, 3+ positive; TTP, time to positivity; IFN- γ, interferon gamma.

Proportion QFT-GIT positive at different time points per group (Figure 8)

The percentage of QFT-GIT-positive patients in the TB-no-ART group between month 0 (81%) and 3 (56%), 6 (50%) and 12 (46%) was not significantly different (p=0.10, 0.07 and 0.06, respectively, for comparisons for each time point relative to the baseline). The pattern was similar in the TB-ART group between month 0 (80%), 3 (73%), p = 0.50; however significant differences were detected when month 0 was compared to month 6 (54%) and 12 (50%), (p=values of 0.04 and 0.02 respectively). When the proportion of QFT-GIT-positive in the no-TB-ART group at baseline (26%), was compared to month 3 (68%), 6 (67%) and 12(76%), an increased proportion of individuals were positive, p = 0.01; 0.02; and 0.004 respectively.

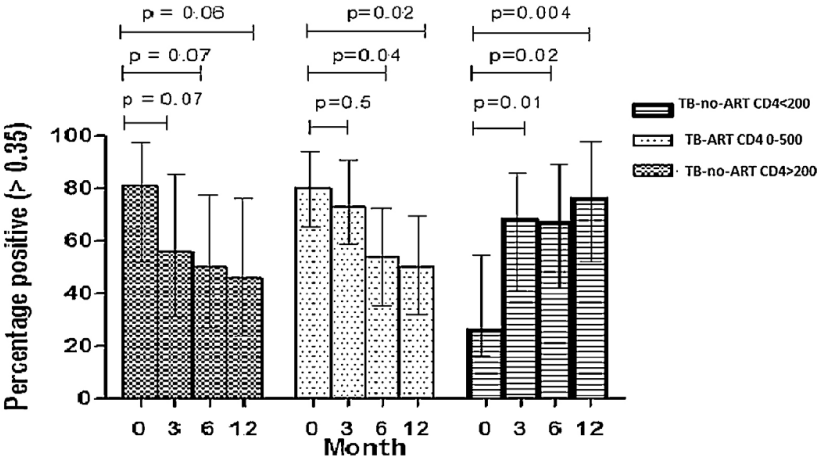

Figure 8. Proportion of patients with a QuantiFERON-TB Gold In-Tube result >0.35 IU/ml by group.

The effect of ART on IFN-γ responses (Figure 9 and Figure 10)

Change between month 0 and month 3

With regard to changes in median IFN-γ responses depending on ART or not, irrespective of TB culture status at baseline (Figure 11 and 12); TB-ART participants, n=30, had an IFN-γ median response at month 0 and 3, of 1.05 vs. 3.76 IU/ml, a per patient median increase of 0.015; IQR -0.22:2.4, p=0.07. In TB-no-ART participants (n=17), IFN-γ median responses were 2.1 and 0.895 IU/ml for these time-points and there was a per patient median decrease, -0.27 (IQR -2.4:2.4, p=0.50). In Non-TB-ART participants (n=18), IFN-γ median response was 0.14 vs. 1.7 IU/ml and there was a median increase was 0.98; IQR 0-7, p=0.004. Figure 12 shows a per patient variation in QFT-GIT changes, with the participants receiving ART showing more elevations while those not receiving ART show more declines.

Change between month 0 and month 6

There was no change in median IFN-γ responses from month 0 to month 6 in TB-ART group, IFN-γ responses were 1.05 vs. 0.7 IU/ml, and there was a median per patient decrease of 0.11 (IQR -0.65:0.19), p=0.30. In TB-no-ART group, IFN-γ median response was 2.1 vs. 0.47 IU/ml and there was a median per patient decrease was -0.27 (IQR -2.2:0.75), p=0.20. In the Non-TB-ART group, the IFN-γ median response was 0.15 vs. 1.5 IU/ml and there was a median per patient increase was 0.63; IQR 0:2.6, p=0.008.

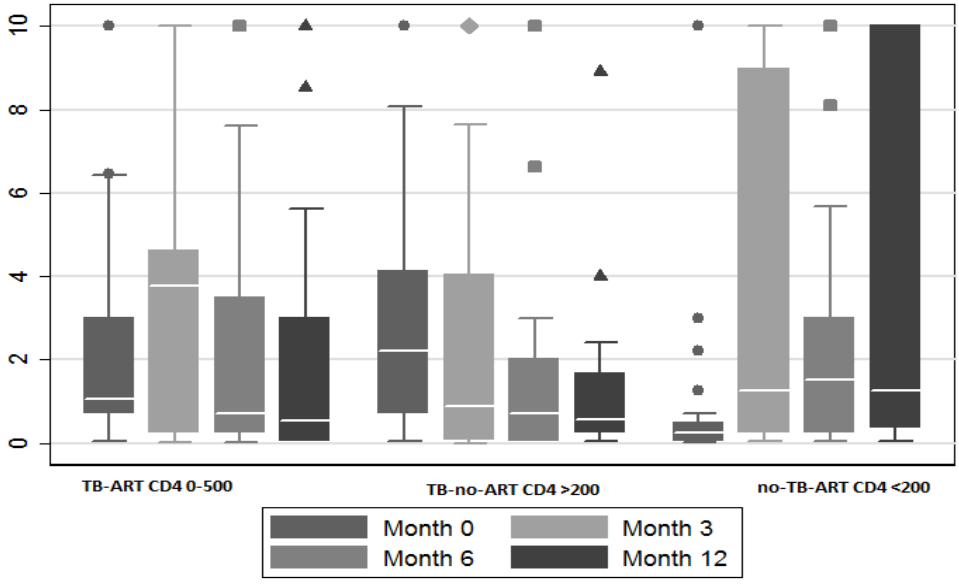

Figure 9 Longitudinal IFN-γ responses are shown for patients undergoing ATT (n=17) and those undergoing TB treatment and ART (n=30) at 0, 3, 6 and 12 months, as well as for non-TB patients on ART (n=18).

Change between month 0 and month 12

The only significant changes were in No-TB-ART group, with a median IFN-γ 0.14 vs. 1.25 IU/ml and a median per patient increase of 0.95 (IQR 0-7, p=0.03). The rest of the changes were similar to those between month 0 and 6.

Longitudinal changes in IFN-γ responses

The change in IFN-γ responses from baseline through to month 12 varied extensively, although in general there was a decline from month 6 to 12. In the groups receiving ART, for participants with high baseline levels (2.0 and above), there was a steady decline to month 12. The participants with lower levels (below

2), there was a sharp increase of IFN-γ levels at month 3, followed by a sharp decline at month 6 and a steadier decline to month 12, which was seen less in the group not receiving ART.

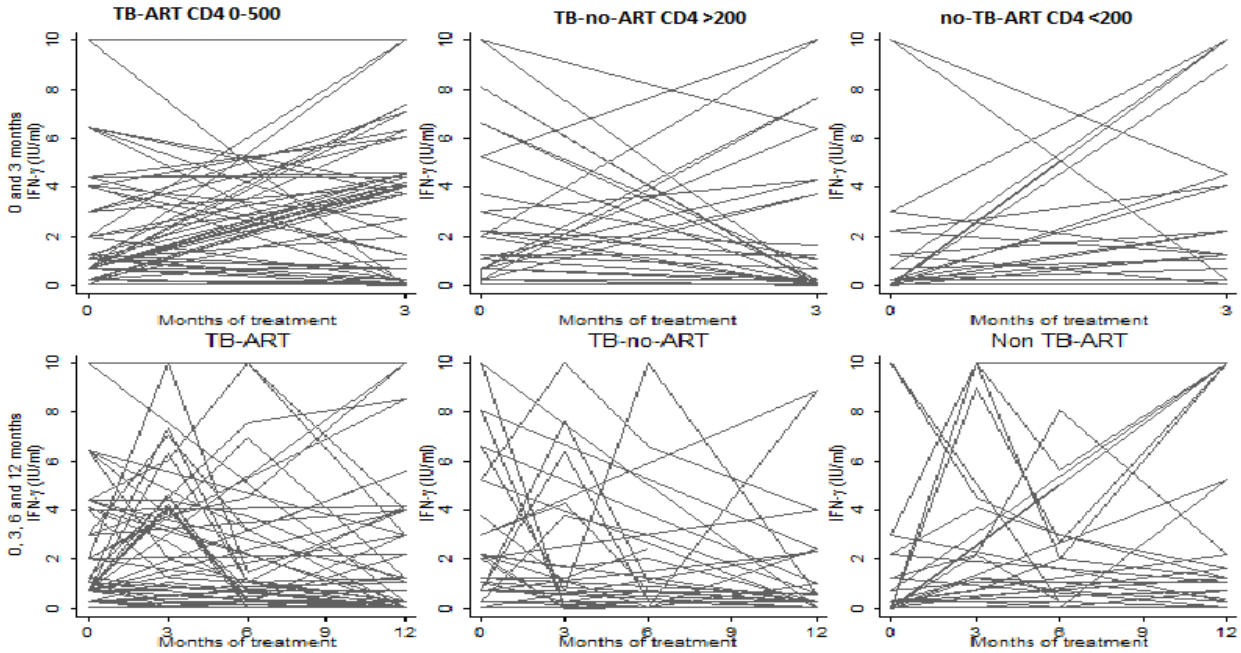

Figure 10 Longitudinal changes in QFT-GIT: TB patients on ART, n=30; TB patients not receiving ART, n= 17 and Non-TB patients on ART, n=18 between 0 and 3 months and between 0, 3, 6 and 12 months.

Longitudinal changes in sputum culture status and QFT-GIT responses

Median IFN-γ responses from month 0, 3 and 6 irrespective of ART did not differ between TB participants who culture converted at month 2 and those who did not; 1.25 vs.1.05, 3.76 vs. 1.15 and 0.06 vs. 0.7 respectively. Median IFN-γ responses from month 0, 3 and 6 did not differ significantly between the non-TB-ART participants who remained culture negative versus those who converted to positive: 0.15 vs. 0.47, 1.73 vs. 1.6, and 2 vs. 0, respectively. Only three participants remained culture positive (either solid or liquid) at month 6, (reduction from 1.12 to 0.06IU/ml; p=0. 02). All other participants in the TB and No-TB-ART groups were culture-negative at month 6. Four previously smear and culture negative participants (no-TB group) converted to culture (MGIT or 7H11) positive at month 2, median IFN-γ increase from 0.47 to 1.6 IU/ml, p=0.08, three received ATT. Two participants were culture positive at month 6, both received ATT, IFN-γ declined from 0.15 to 0IU/ml. When the "grey zone" criteria (0.2-0.7 IU/ml) was applied (45), of the 18 non-TB-ART participants, 9 had baseline QFT < 0.2, of whom three remained below 0.2 at month 3, and six increased to above 0.7. Two remained below 0.2 at month 6; two increased to the "grey zone" and five increased to above 0.7. Five were in the "grey zone" at baseline: two

increased to > 0.7 by month 3 and were treated (culture positive); three remained in the "grey zone" (not treated). Four participants were > 0.7 at baseline and remained > 0.7 at month 3 and 6 (one treated from culture results). Patients were not treated if no bacterial evidence existed.

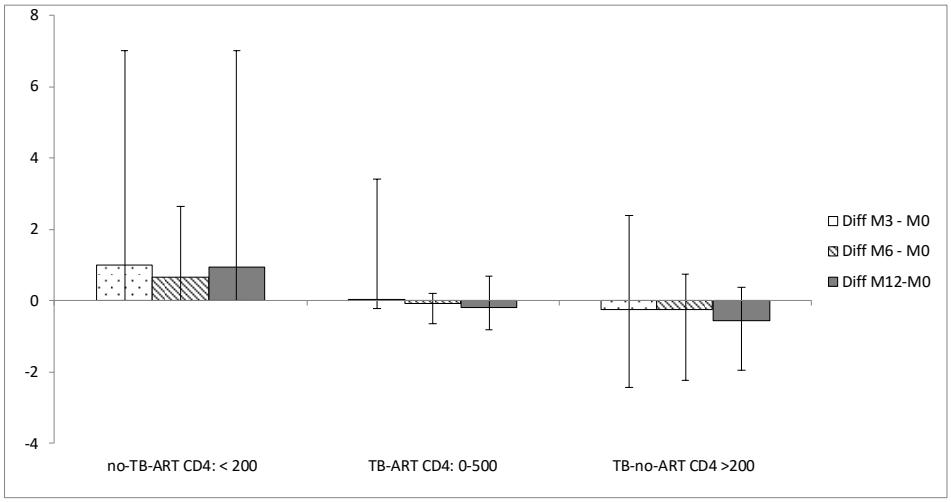

Figure 11 Median change with error bars for 3 groups: no-TB-ART, TB-ART and TB-no-ART.

Discussion:

There are limited or no data related to how quantitative RD-1-specific T-cell responses change during the course of treatment (ART or TB treatment) in HIV-TB co-infected persons. There are also hardly any data about how such responses correlate with microbiological readouts in patients on TB treatment, whether HIV-infected or not. The key findings of this paper are that: (i) overall, although T-cell responses generally declined with ATT, IFN-γ responses (magnitude or reversion) did not correlate with studies of bacterial load at diagnosis or with culture-conversion at 2 to 3 months post treatment initiation; (ii) in contrast to TB treatment alone, ART in HIV-TB co-infected persons, caused a transient increase in T-cell responses which then fell over time to levels similar in patients not receiving ART; and (iii) ART alone in HIV-infected persons without TB resulted in a gradual increase in T-cell responses over several months. Thus, ART must be taken into account when evaluating T-cell responses (e.g. IGRA and by inference TST) in HIV-infected persons irrespective of whether they are on TB treatment, and although such responses generally decline with ATT there is no correlation with 2-month culture conversion readouts.

This study confirmed the findings of Theron et al. and that of Oni et al. studies from a high burden setting reporting the correlation of serial antigen-specific T-cell responses/ IGRAs with bacterial load (40, 46).

Thus, with the exception of this report and the findings reported here, studies from either low- or high-burden settings have not previously reported the correlation of IGRA responses with measures of mycobacterial burden (8, 12, 13, 16, 18, 20-23, 26, 28, 32, 37, 47, 48). Although culture conversion in itself has sub-optimal predictive value for a favorable treatment response, the lack of any correlation, and no difference in the magnitude of IGRA responses in microbiologically positive or negative persons, makes it unlikely that IGRAs will be useful as treatment monitoring tools or prognostic markers in HIV-TB co-infected persons (or uninfected persons with TB for that matter). The lack of correlation is not surprising because antigen-specific memory T-cell readouts cannot distinguish between exposure, latent infection, and active TB(14, 49).

In contrast to TB treatment alone, ART in TB-HIV co-infected persons caused a transient spike in T-cell responses followed by a declining response thereafter, and this pattern was independent of CD4 count (though ostensibly more robust in those with a CD4 count of less than 200 cells/ml). This is, to our knowledge, the first report of how serial IGRA responses change in HIV-TB co-infected patients on ART versus no ART. The cause for the transient increase in responses is unclear but factors likely to be important include: (i) immune reconstitution due to the treatment of TB itself (down-regulation of an anti-inflammatory response driven by cytokines such as interleukin 10 and cell types including regulatory T-cells (49-51), (ii) the immune reconstitution effect of ART, which causes the redistribution of effector memory T-cells to the blood compartment, and (iii) release of TB antigen consequent upon treatment which may augment T-cell responses. Given the lack of this spike-like response in the HIV-TB without ART group suggests that ART is the major driver of this phenomenon. TB immune reconstitution inflammatory syndrome (TB-IRIS) is also associated with exaggerated cytokine responses including antigen-specific Th1 responses, although there was no difference in participants on TB treatment and ART who had TB-IRIS versus those that did not (52). These observations have important implications for the interpretation of serial IGRA responses in HIV-infected persons. The treatment of presumed latent TB-infection in children resulted in a similar transient exaggerated response (34).

ART alone (without active TB) resulted in a significant increase in IGRA responses by month 3. This is likely due to ART-related immune reconstitution and redistribution of effector memory T-cells back into the blood compartment (53). Similarly, ART can cause apparent conversion of the TST whereby TST-negative persons become TST-positive on ART (54, 55). These data have important implications for

interpretation of immunodiagnostic tests in the context of IPT provision in high burden settings as current data suggest that only TST+ve persons are likely to benefit from IPT (56, 57).

There are several limitations to this work, including the small number of patients and the fact that QFT-GIT is not the most suitable test for measuring IFN-γ. However, although there was a decline in evaluable participants due to deaths and withdrawals, and the number of participants without complete results for all time points decreased, there was still sufficient data to show interesting information on the effects of concomitant TB-HIV treatment, and much needed data related to its impact on serial IGRA responses are provided. This study further sheds light on the impact of the CD4 count in this context, (although the study was conducted during a time when newer and more effective ART was unavailable within the national treatment programme). However, even with this limited dataset that had limited power, no trend or relationship between bacillary load at diagnosis and IFN-γ reversion (0 to 3 months) or sputum culture conversion could be shown. It should be borne in mind that the predictive value of culture conversion itself for a long-term favorable outcome (failure or relapse at 2 years) is sub-optimal (58). However, the lack of correlation of initial and serial IGRAs with bacterial load and high proportion of HIV-TB cases remaining IGRA positive at 6 and 12 months post treatment makes it unlikely that it would make a good marker of treatment response. Chiappini et al. concluded that monitoring IGRA changes over time seems to have only speculative value in adults (59). This meta-analysis however did not differentiate between those who were on concurrent ART or no ART.

In conclusion, these preliminary data suggest that although QFT-GIT readouts decreased with time during ATT, irrespective of whether participants were on ART, they did not correlate with 2- or 6-month culture-conversion and markers of bacillary burden. Therefore, IGRAs do not appear to be useful for the prognostication of treatment outcome in co-infected patients on concurrent therapy.

Competing interests

There were no competing interests declared by any of the authors.

Authors' contributions

TM participated in design, managed the study and drafted the manuscript. RR and AP participated in the design and management of the study, CC performed the statistical analysis, GT helped draft the manuscript

and KD participated in design of the study and helped to draft the manuscript and its final edit. All authors read and approved the manuscript.

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Ethics approval was obtained from Biomedical Research Ethics Committee

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Chapter 5

Longitudinal assessment of health-related quality of life of HIV-infected patients treated for tuberculosis and HIV in a high burden setting

Introduction

Treatment monitoring of TB and HIV is necessary but the most used method is biomedical. Since TB affects the psychosocial aspects of patients, it is important to use a biopsychosocial model when assessing treatment response. Quality of life assessments add the psychosocial aspects to this evaluation. Very few studies have evaluated their use in co-infected patients especially in high TB burden areas.

This section addresses objective 3 of this thesis.

Objective 3 - To evaluate HRQOL in TB-HIV co-infected patients receiving combined treatment.

Sub-objective 3.1 - To examine changes in the HRQOL of patients receiving TB-HIV co-treatment.

Sub-objective 3.2 - To evaluate whether HRQOL differs in patients receiving TB treatment alone or together with ARV therapy.

Sub-objective 3.3 - To compare changes in the HRQOL of co-infected patients receiving TB-HIV co-treatment with those receiving TB treatment alone.

The manuscript below addresses the above objective. The present study assessed HRQOL of patients co-infected with TB and HIV at different levels of immunosuppression as well as in HIV-infected patients without TB with CD4 count below or equal to 200 cells/mm^3. The manuscript details the HRQOL from baseline to 12 months after start of treatment. The analysis shows whether patients in combined treatment tolerate treatment. Reversed items were adjusted such that the higher the score the better the HRQOL was for all subscales. AEs and SAEs reported by patients (and not laboratory AEs and SAEs) were considered for the relationship with HRQOL.

Longitudinal assessment of health-related quality of life of HIV-infected patients treated for tuberculosis and HIV in a high burden setting

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Abstract

Introduction: Assessment of patients receiving treatment for human immunodeficiency virus (HIV) and tuberculosis (TB) using a Health Related Quality of Life (HRQOL) instrument is important to get the subjective view of the patients' wellbeing.

Methods: We used the Functional Assessment of HIV Infection (FAHI) HRQOL instrument to collect perceived wellness information at baseline, month 3, 6 and 12 from patients enrolled in a pharmacokinetic study between March 2007 and April 2008. Composite domain scores at each time point and their relationship with the rate of adverse events(AEs) and serious adverse events (SAEs) were compared between treatment arms.

Results: Out of the 82 patients enrolled, 76 were analysed. There was a significant increase in total score in all groups between baseline, month 3, 6 and 12 (all p-values<0.0001), and over time (p<0.001). Adjusting for baseline total score, baseline CD4 count had a significant effect on the total score (p= 0.002) and the rate of change in total score over time, that is; interaction effect (p<0.001). There was no difference in each domain scores between participants that received ART with TB treatment and those that received TB treatment only. Respiratory AEs had a significant effect on HRQOL.

Conclusion: We found that assessment of HRQOL of participants in TB-HIV treatment using the FAHI instrument was useful in evaluating treatment responses. It showed improvement consistent with decrease in adverse events and signs and symptoms of TB. Number and type of AEs was related to lower HRQOL in spite of TB cure.

KEYWORDS: Health Related Quality of Life, tuberculosis, HIV, adverse events

Background

Quality of life (QOL) evaluation has been conducted for decades especially in cancer research. There is an increasing trend to include QOL in clinical trials, but this is still lacking in TB-HIV research. QOL has been defined as how well people are able to perform activities of daily living and their well-being (1), the health effects of QOL led to the introduction of the term Health Related Quality of Life (HRQOL) by Kaplan and Bush (2).Different studies have investigated HRQOL in participants receiving treatment for TB (3-9) or participants receiving therapy for HIV(10-17), but studies investigating the HRQOL of patients co infected with TB and HIV and receiving concomitant therapy are limited (1, 18-20), and are mostly cross sectional studies. Of the 22 high burden TB countries listed by the World Health Organization (WHO), South Africa is ranked third globally. TB incidence estimates reported in 2013 shows a slight decrease from 430 000-630 000 in 2012 (21) to 410 000-520 000 (22). South African estimates for HIV-infected incident cases decreased from 270 000-390 000 in 2012 to240 000–310 000 in 2013. More than 70% of patients with TB in South Africa are also HIV-infected (23), and TB is the leading cause of HIV related morbidity (24). It is well established that Highly Active Antiretroviral Therapy (HAART) reduces opportunistic infections as well as reducing morbidity and mortality (25, 26). In order to evaluate the benefit of ART in TB-HIV patients, HRQOL could be used.

The probability of problems related to side effects when receiving TB may be worsened by concomitant HIV therapy, thereby affecting QOL treatment (27). Patient-reported QOL may help clinicians decide how to proceed with the patient's treatment if assessed using a valid and reliable instrument. It is recommended by Dhuria et al. that a multidimensional instrument is used (9).

The aim of the study was to compare the HRQOL of TB and HIV co infected patients receiving TB treatment alone and those receiving treatment for both conditions at the same time. We also evaluated HRQOL of HIV-infected patients without TB receiving ART. The current study was undertaken to examine changes in HRQOL after initiating TB treatment and ARVs, TB treatment alone and ARVs alone over a 12-month period. The study was part of a pharmacokinetic study of anti-tuberculosis and antiretroviral therapy.

Methods

Setting

This study was conducted in Durban, South Africa. All patients were HIV positive or co- infected with TB and recruited from clinics around Durban between March 2007 and April 2008.

Study design

This was a sub-study to a randomised control trial (RCT), where patients were randomised into early HAART or no HAART depending on their baseline CD4 count.

Participants and inclusion criteria

HIV positive patients aged 18 to 65 years, with and without TB, who provided written informed consent, were included in the study (28). Patients were excluded if they had a history of drug resistant tuberculosis or TB treatment in the past 2 years, an indication for medication that might interact with study treatment, treatment dependent diabetes mellitus, epilepsy, a history of excessive alcohol consumption or drug abuse, pregnant women and women of childbearing age refusing to use standard barrier contraceptive measures or intra uterine device (IUD) for the duration of the study. Patients with severe illness, weighing <30 kg, with hepatic transaminases >2.5 times the upper limit of normal (ULN), serum creatinine >1.5 times ULN, neutrophils <1200/mm^3 or haemoglobin <8 g/dL were also excluded.

Participants were enrolled when they had TB/HIV co-infection and their CD4 count was above the threshold for commencement of ARVs according to the national programme (above 200 during the study period). Also, TB patients co infected with HIV with CD4 count below 200 were enrolled and commenced on early ARVs (within two weeks of starting TB treatment). HIV-infected patients without TB, with CD4 count below 200 were also included in the study. Four groups were recruited: high CD4 count on TB treatment and ART(CD>200TH); high CD4 count on TB treatment alone (CD>200T); low CD4 count on TB and HIV therapy (CD≤200TH) and low CD4 on non-TB on HIV therapy (CD≤200H).

Procedures

All patients were asked to choose a directly observed therapy (DOT) supporter before starting treatment. TB was diagnosed by sputum smear to confirm TB-HIV co-infection. Assessments were conducted at baseline and repeated monthly for the first six months. They included a physical examination, full blood count, urea and electrolytes, liver transaminase assays (aspartate transaminase [AST] and alamine aminotransferase [ALT]). After 6 months these assessments were conducted every 3 months until 24 months, i.e. study completion. Information on adverse events was collected at each visit for 24 months.

Clinical and abnormal laboratory AEs were graded according to the Division of AIDS Table for Grading the Severity of adult and paediatric Adverse Events (DAIDS AE Grading Table), grade 3 and 4 adverse events were reported as serious adverse events (SAEs).

Treatment

All TB patients received fixed dose combination standard anti-TB treatment (ATT) comprising of isoniazid, rifampicin, pyrazinamide and ethambutol for two months and fixed dose combination treatment comprising isoniazid and rifampicin for 4 months. All participants with CD4 count below 200 without TB were started on ART immediately after enrolment, efavirenz and combivir (lamivudine and zidovudine) and on the 14th day of enrolment for the CD4≤200 TB-HIV group. The CD4>200 group with TB-HIV was randomized into group that received ART on 14th day post randomization or no ART.

Patients completed the Functional Assessment of HIV Infection (FAHI) before treatment commencement, at month 3 (after end of intensive phase), at month 6 (at the end of TB treatment), and at month 12. The core of the instrument is a general HRQOL instrument with an additional subscale for mental wellbeing. The domains of FAHI include physical (score 0-40), functional (score 0-52), social (score 0-32), emotional (score 0-40) and cognitive wellbeing (score 0-12), with a maximal total score of 176 (29), obtained by totaling all subscale scores. Lay counselors were trained on how to administer the FAHI Zulu language questionnaire by interviewing patients and filling the responses. Professional nurses were trained on the administration and checking the filled questionnaires for completeness. Where there were errors, they were sent back to the counselor who did the correction. If clarification was needed from the patient this was sought as the patient was admitted for the day and was available to answer questions. The questionnaires were then double entered into Epidata. Data cleaning was conducted by query generation by data capturers and where there were discrepancies, these were sent to the professional nurse who ensured that they were resolved.

We included 10 additional questions on: waking up during sleep, abnormal dreams, feeling sleepy during the day, alcohol consumption, balance, dizziness, hearing, painful feet, numbness of feet, and body shape changes. These ten questions were scored from 0-4 therefore the score total ranged from 0-40. We selected these questions based on the known side effects of efavirenz and piloted them on 10 patients receiving ART. These patients found them easy to respond to and easy to understand. The sign of the item test

correlations were examined to determine whether an item in the scale needed to be reversed or not.

Statistical analysis

The Cronbach's alpha was used to measure internal consistency of the individual score components for each domain. Demographic characteristics were presented as frequencies and percentages for categorical variables, and as means with standard deviations for continuous variables. Skewed variables were presented as medians with interquartile ranges (IQRs). Comparisons between treatment arms at baseline were performed using the Chi-squared test and Fishers exact test for nominal variables, and non-parametric trend tests for ordinal variables. T-tests were used to compare means and the Wilcoxon rank sum test was used to compare the distribution of skewed variables between groups.

All analysis was done in accordance with intention to treat (ITT) principles. The individual domain scores and total score at each time point (Baseline, month 3, 6 and 12) were compared between 1) CD>200TH and CD>200T 2) CD≤200TH and CD≤200H and 3) CD>200 and CD<=200 using unpaired t-tests, unless otherwise stated. To test the aforementioned differences after adjusting for baseline scores, ANCOVA (analysis of covariance) was used. All comparisons were planned and no adjustment was made for multiple comparisons. To compare the overall trend of quality of life scores over time, linear mixed models with random intercepts and slopes were fitted.(30) Nested models were compared using the likelihood ratio test and information criteria were used to select the model which provided the best fit to the data. The number of adverse events was compared between the four groups using the Kruskal-Wallis test, and pairwise comparisons were performed using the Wilcoxon rank sum test. The association between HRQOL scores and the type of adverse event experienced in the preceding period was assessed separately at each time point using median quartile regression. We used the standard error of measurement (SEM) to define important change in the total quality of life score (31, 32).All analysis was performed in Stata version 13(StataCorp, College Station, TX, USA).P-values less than 0.05 were considered statistically significant.

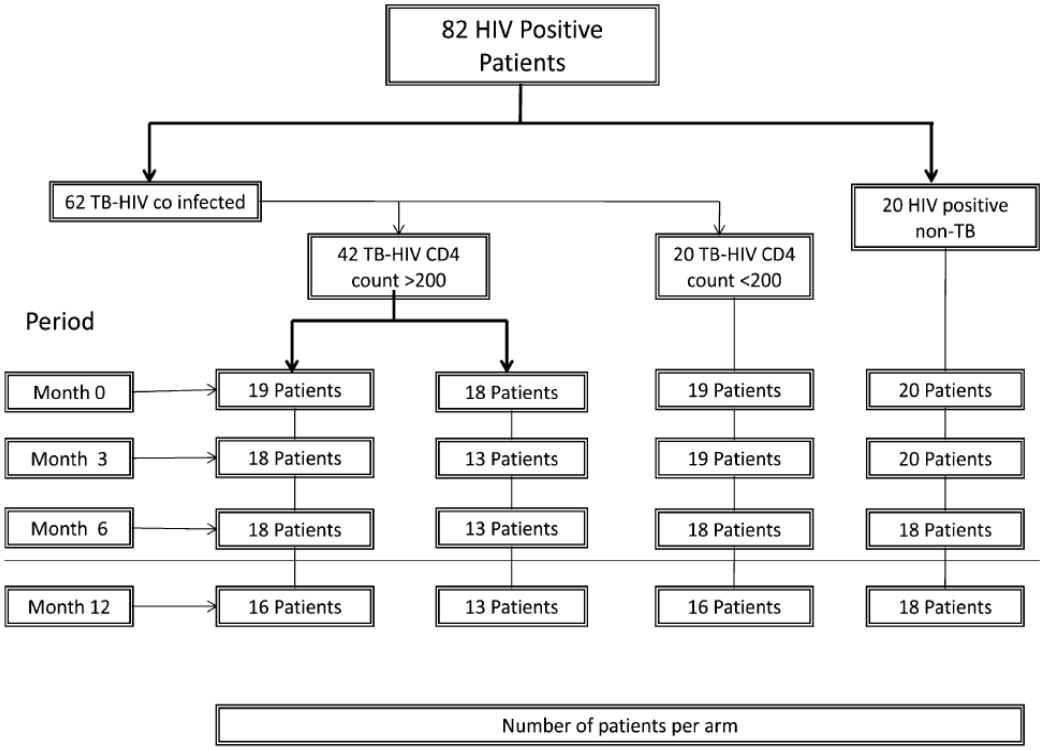
Results

Baseline characteristics

A total of 82 participants were enrolled, Figure 12 is the flow chart showing the number of patients analysed from baseline to month 12. Seventy-six participants were analysed, and their baseline characteristics are presented in Table 14. Age and night sweats were significantly different between the CD4>200 and ≤200 groups. The remaining characteristics were similar between groups within the CD4 count categories, with

no statistical significance noted. Age was statistically significant between CD4 count >200 and CD4 count ≤200 groups.

Figure 12. Patient flow chart



ATT anti tuberculosis treatment, ART antiretroviral therapy

Treatment outcomes

Six participants had been withdrawn by month 3, and refused follow up, 70 remained. Three participants died before month 6, 67 remained. Two participants died before month 12, 1 MDR-TB and 1 had no questionnaire, 63 remained.

There were 7 deaths in the study, 5 before month 12. One of these was in the CD4>200 while 6 were in the CD4<=200 group. Two non-TB participants converted to active TB within the first 3 months and were started on ATT. One became multi-drug resistant and was withdrawn and sent for treatment at month 9. Only 4 out of six participants were smear or culture positive at month 5 or 6, 4 of which were in the CD>200 group and 2 in the CD<200 group.

Table 14 Baseline Demographic Characteristics

		CD4>200			CD4 ≤200			p-value
		TB+ARV therapy	TB treatn only	P-value	TB+ARV therapy	ARV ther only	P-value	
		N=18	N=19		N=19	N=20		
Sex	Female	10 (55.6%)	7 (36.8%)	0.25	12(63.2%	11(55.0%)	0.61	0.25
Age (M (SD))		29.8 (7.5)	31.6 (6.5)	0.46	36.1 (7.2)	35.0 (7.1)	0.62	0.004
Median C count (IQR)		310 (274 - 333)	318 (242 - 345)	0.77	110 (68- 159)	108 (72.5 -159	0.80	
TB sympton								
Cough	Normal	4 (22.2)	6 (31.6)	0.361	3 (15.8)			0.118
	Mild	9 (50.0)	10 (52.6)		8 (42.1)			
	Moderate	5 (27.8)	3 (15.8)		8 (42.1)			
Night sweats	Normal	12 (66.7)	6 (31.6)	0.062	4 (21.1)			0.019
	Mild	4 (22.2)	10 (52.6)		8 (42.1)			
	Moderate	2 (11.1)	3 (15.8)		7 (36.8)			
Chest pain	Normal	10 (55.6)	13 (68.4)	0.480	9 (47.4)			0.300
	Mild	6 (33.3)	4 (21.1)		7 (36.8)			
	Moderate	2 (11.1)	2 (10.5)		3 (15.8)			
Fever	Normal	9 (69.2)	12 (70.6)	0.596	10 (52.6)			0.268
	Mild	4 (30.8)	3 (17.7)		8 (42.1)			
	Moderate	0 (0.0)	2 (11.8)		1 (5.3)			
Haemoptysis	Normal	18 (100)	19(100)		18 (94.7)			0.163
	Mild	0	0		1 (5.3)			
	Moderate	0	0		0			

Quality of life outcomes

Reliability and validity

The Cronbach's alpha, used to measure internal consistency, was 0.90 for the physical domain; 0.89 for the emotional domain; 0.86 for the functional domain; 0.87 for the social domain; 0.55 for the cognitive domain, and 0.79 for the CNS domain. Average value of inter-item correlations were used to assess reliability, the value of which was 0.72 for the cognitive and social domains and 0.63, 0.71, 0.68 and 0.58 for the functional, emotional physical and CNS domains, respectively.

Total HRQOL scores between groups

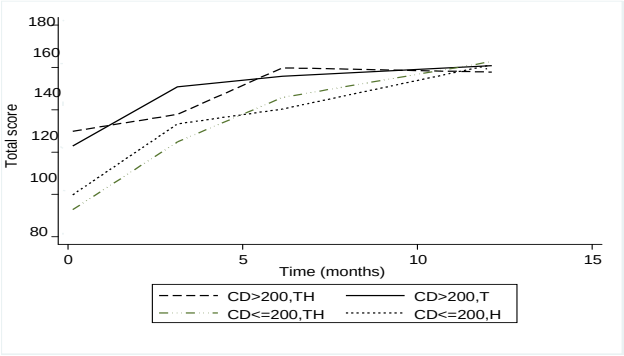
Table 15 shows total scores over time for the treatment groups. At baseline there was a significant difference in the HRQOL between the four groups (p= 0.005). At month 3 there was a significant difference between HRQOL of the 4 groups; p= 0.003.

Table 15: Mean domain and total scores of FAHI and CNS at different time points

Domain	Time	CD > 200			CD ≤ 200			*p* value	*p* value adjusting for baseline
		CD > 200TH Mean (SD)[1]	CD > 200T Mean (SD)[2]	*p* value	CD ≤ 200TH Mean (SD)[3]	CD ≤ 200H Mean (SD)[4]	*p* value		
Physical	Month 0	26.50 (8.7)	28.05 (11.0)	0.64	23.25 (9.8)	23.40 (10.3)	0.96	0.08	N/A
	Month 3	34.69 (4.8)	35.22 (5.5)	0.78	30.58 (8.3)	28.50 (10.1)	0.49	<0.01	<0.01
	Month 6	38.69 (1.6)	36.06 (8.6)	0.28	34.94 (5.7)	34.17 (7.0)	0.72	0.09	0.14
	Month 12	38.00 (3.2)	38.56 (3.8)	0.68	39.69 (0.6)	35.94 (8.2)	0.08	0.74	0.65
Emotional	Month 0	22.56 (11.4)	25.48 (11.0)	0.43	17.42 (9.9)	19.10 (10.8)	0.62	0.02	N/A
	Month 3	29.85 (7.9)	32.61 (6.5)	0.29	24.68 (8.0)	25.90 (8.4)	0.65	<0.01	0.01
	Month 6	33.46 (6.5)	32.00 (10.2)	0.65	29.56 (8.5)	30.67 (6.3)	0.66	0.26	0.41
	Month 12	33.74 (8.9)	33.84 (8.3)	0.97	38.00 (2.7)	33.56 (8.6)	0.06	0.25	0.30
Functional	Month 0	41.33 (8.6)	38.42 (11.5)	0.39	29.11 (10.0)	33.50 (11.9)	0.22	<0.01	N/A
	Month 3	40.69 (8.8)	44.89 (8.7)	0.20	36.68 (8.7)	36.65 (10.9)	0.99	<0.01	0.07
	Month 6	45.31 (7.1)	42.22 (10.4)	0.36	43.61 (5.5)	40.72 (7.4)	0.19	0.03	0.58
	Month 12	43.93 (7.3)	43.44 (5.3)	0.83	45.94 (5.5)	44.06 (8.2)	0.44	0.96	0.27
Social	Month 0	24.06 (7.4)	22.89 (8.2)	0.65	18.26 (7.9)	19.90 (10.8)	0.59	0.03	N/A
	Month 3	24.19 (6.2)	26.94 (4.18)	0.15	23.54 (5.3)	20.95 (8.6)	0.27	0.01	0.10
	Month 6	24.58 (8.7)	26.32 (7.4)	0.55	25.03 (5.6)	22.71 (7.7)	0.31	0.21	0.51
	Month 12	28.97 (4.0)	29.55 (2.8)	0.65	28.93 (3.9)	27.21 (8.2)	0.45	0.17	0.54
Cognitive	Month 0	10.00 (2.8)	9.63 (3.0)	0.70	7.47 (3.4)	7.70 (3.1)	0.83	<0.01	N/A
	Month 3	9.31 (2.8)	9.78 (2.6)	0.64	7.89 (2.6)	7.85 (2.8)	0.96	<0.01	0.09
	Month 6	10.77 (1.9)	10.17 (2.0)	0.40	8.78 (2.6)	8.78 (2.6)	1.00	<0.01	0.02
	Month 12	8.85 (2.6)	9.50 (2.3)	0.47	10.00 (2.1)	9.78 (2.4)	0.78	0.30	0.08
Total score[a]	Month 0	124.4 (29.8)	124.5 (29.1)	0.91	95.5 (25.8)	103.6 (33.1)	0.40	<0.001	N/A
	Month 3	138.7 (20.3)	149.4 (21.4	0.15	123.4 (27.2)	119.9 (30.1)	0.69	<0.001	0.01
	Month 6	152.8 (19.0)	146.8 (23.8)	0.54	141.9 (23.9)	137.0 (22.6)	0.68	0.05	0.25
	Month 12	153.5 (16.0)	154.9 (17.6)	0.73	162.6 (10.49)	150.5 (29.4)	0.40	0.99	0.39
CNS	Month 0	27.5 (7.25)	28.7 (6.5)	0.60	21.6 (8.4)	24.7 (8.4)	0.27	<0.01	NA
	Month 3	31.6 (3.6)	33.4 (3.3)	0.16	28.9 (4.4)	29.2 (5.1)	0.87	<0.001	<0.01
	Month 6[b]	36 (33–36)	35 (33–36)	0.63	32.5 (31–35)	33 (31–36)	0.95	0.01	0.07
	Month 12[b]	35 (34–36)	36 (35–36)	0.23	35 (33–36)	34 (31–36)	0.37	0.10	1.00

[1] 19, 18, 18 and 16 individuals at month 0, 3, 6 and 12 respectively
[2]18 individuals at month 0 and 13 individuals at time points 3, 6 and 12
[3]19, 19, 18 and 16 individuals at month 0, 3, 6 and 12 respectively
[4]20, 20, 18 and 18 individuals at month 0, 3, 6 and 12 respectively
[a]Total score comprised of Physical, emotional, functional, social and cognitive domain scores
[b]Presented as median with IQR and p-values from Wilcoxon rank sum test and median quantile regression for baseline adjusted comparisons

Figure 13: FAHI total scores from baseline to month12



CD>200,TH: CD4 count > 200, on ATT and ART; CD>200,T: CD4 count > 200 on ATT;
CD<=200,TH; CD4 count <=200, on ATT and ART; CD<=200,H; CD4 count <=200 on ART

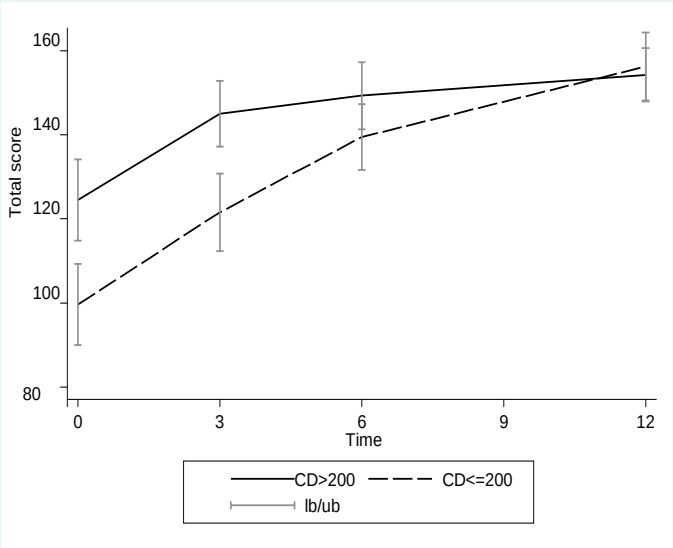
At month 6 and 12, there was no significant difference in HRQOL between the 4 groups; p=0.263 and 0.375, respectively. After adjusting for baseline, month 3 total scores were still significantly different between CD4> and CD4≤200 groups; p=0.01, but not for month 6 and month 12. Baseline scores in the CD4>200TH and CD4>200T groups were similar; p-value=0.99). At month 3, 6 and 12 there were no significant differences in HRQOL, (p = 0.17,0.45 and 0.82 respectively. Table 15 also shows the baseline scores between the CD4≤200,TH and CD4≤200,H groups were not significantly different; p=0.40. Month 3, month 6 and month 12 scores were not significantly different, p = 0.70, p= 0.43 and p=0.13 respectively.

Since no significant differences were found within the higher CD4 count groups, we combined CD4>200TH and CD4>200T to form the CD4>200 group and CD4≤200,TH and CD4≤200,H to form the CD4≤200 group. Table 16 shows mean total scores, sd and p-values for CD4> and ≤200.Between the CD4>200 and CD4≤200 groups, there was a significant difference in the baseline, p<0.001 and month 3 total scores, p<0.001and month 6 total scores, p=0.08 between the CD4>200 and CD4≤200 groups, but not for month 12, p =0.71. After adjusting for baseline total score, there was still a significant difference in the total score at month 3, p= 0.010.

Quality of life trends over time between treatment groups

The longitudinal HRQOL score for the four groups is presented in Figure 12. The change from baseline of the total scores combined was significant from month0 to month12, p<0.0001. Further statistical analysis confirms that the trend is indeed significantly increasing; p<0.0001. Adjusting for baseline total score, baseline CD4 count had a significant effect on the total score, p=0.002, and the rate of change in total score over time i.e. interaction effect; p<0.001).

Figure 14: Change from baseline in HRQOL total score by time



Domain scores comparing CD4>200 and CD4≤200 groups

As seen in Table 15, the baseline physical wellbeing domain scores were not significantly different between groups, p=0.08, however for month 3 there was a significant difference, p=009. At month 6 and 12 the scores were not significantly different, p=0.096 and 0.74, respectively. Baseline and month 3 emotional, functional and social domain scores were significantly different, but month 6 and 12 were not. Baseline, month 3, and month 6 scores were significantly different for the cognitive domain, but not for month 12.

Table 15 also shows the apparent difference between time points on the physical wellbeing scores. There was significant increase in physical, emotional, functional and social wellbeing scores over time: p<0.001 for each domain. However, the mean cognitive wellbeing score did not differ significantly between baseline and month 3 (p=0.86). The largest change in cognitive wellbeing score occurred between month 3 and month 6 (mean difference 0.81, p <0.002).

Table 16: Total score at month 0, 3, 6, 12 expressed as mean(sd)

Month	CD4<=200	CD4>200	p-value
0	99.65 (29.68)	124.46 (29.02)	<0.001
3	121.57 (28.40)	144.94 (21.30)	<0.001
6	139.48 (23.043)	149.30 (21.78)	0.08
12	156.20 (23.09)	154.26 (16.60)	0.71

The CNS domain was similar to the other domains, showing no significant difference between CD4>200TH and CD4>200T as well as between CD4≤200TH and CD4≤H, but significant difference between the CD4>200 and CD4≤200 at baseline, month 3 and month 6 and similar to the cognitive wellbeing domain, was not significantly different at month 12.

Serious adverse events (SAEs), adverse events (AEs) and quality of life

We assessed the presence of SAEs between the following intervals; 0-3, 3-6 and 6-12 months. Twenty-one (28%) patients experienced at least one SAE between 0 and 3 months. There were 11 (15.28%) and 8 (12.7%) patients who experienced an SAE between 3-6 or 6-12 months, respectively. In the above figures, the denominator was all patients in the study at the end of the relevant interval. The number of AEs are shown in Table 17. There was a significant difference in the total number of AEs (SAEs included) between month 3 and month 6 in all groups. For all groups, the median HRQOL total score increased sharply while the median number of AEs decreased sharply from month 3 to month 6 (Figure 15). There was a slight increase in the number of AEs between month 6 and month 12 in the higher CD4 groups.

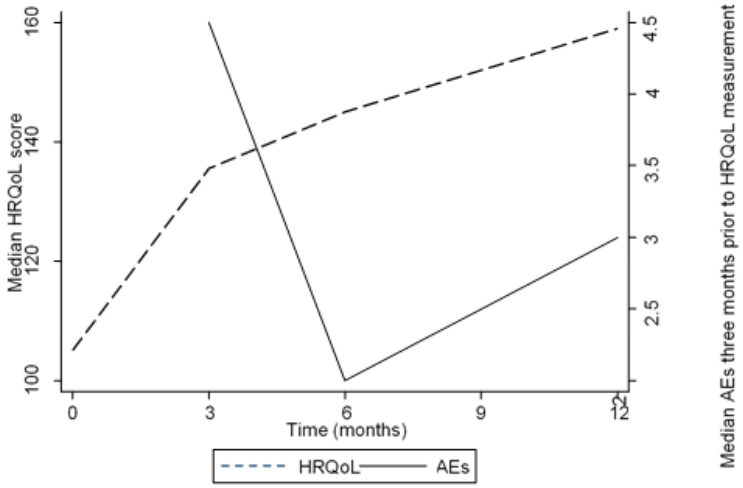
Table 17. Median (IQR) number of AEs per period by arm

ARM	Time 0-3	Time 3-6	Time 6-12
CD>200,TH	3 (2-5)	2 (0-2)	4 (0-5)
CD>200,T	4 (3-7)	2 (1-3)	4 (1-7)
CD<=200,TH	7 (5-12)	3 (1-3)	2 (1-3)
CD<=200,H	4.5 (2.5-7)	2 (1-3.5)	1 (0.5-3)
p-value	0.47	0.04	0.20

TB therapy + ART=TH; TB therapy only=T; ART only=H; adverse event=ae;

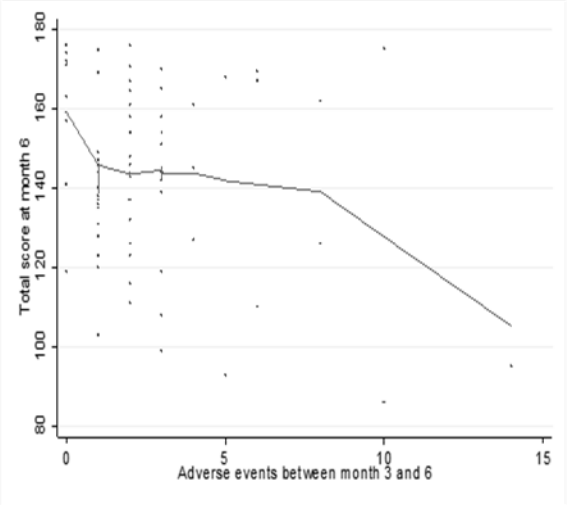
Since the majority of patients had TB we checked the effect of anaemia and respiratory adverse events on HRQoL, as anaemia is one of the common AEs in TB patients (33).The presence of anaemia in the previous period did not have an effect on the mean HRQOL total score in the follow up period, p=0.63, 0.33 and 0.16 for month 3, 6 and 12, respectively.

Figure 15 The relationship between HRQOL and number of AEs in the previous three months



When analysing respiratory AEs we found that individuals who had a respiratory AE between baseline and month 3, month 3 and month 6 and month 6 and month12, had a significantly lower quality of life at the next follow up period; month 3; p=0.0085, month 6; p=0.019 and month 12; p=0.017 respectively.

Figure 16 Adverse events and quality of life between month 3 and 6.



Meaningful decline in HRQOL scores

Minimal important change as defined in the method section, was a change larger than the SEM. For the total score, taking all components into account, Cronbach's alpha was 0.908. The standard deviation at baseline was 31.72 for the total score, for all groups combined, leading to a SEM of 9.62. Minimal important

change in total score was hence defined as a change greater than 9.6 between visits. Changes between scores were calculated as follows: Change from baseline to month three, change from month 3 to month 6 and change from month 6 to month 12. Twenty two patients (28.9%) had decline in HRQOL scores at any time. There were no significant differences between CD4≤ and CD4>200; p=0.51, or between CD4>200TH and CD4>200T, 5(27.8%) and 7(36.8%) patients had decline in HRQOL respectively; p=0.72. There were no significant differences between CD4≤200,TH and CD4≤200H, 4(21.1%) and 6(30%) patients had decline in HRQOL respectively; p=0.72.

Discussion

This study assessed the HRQOL lower in TB/HIV co-infected patients with CD4 count ≤200, as well as in HIV non-TB patients with CD4 count ≤200, this is consistent with available literature (14, 34, 35). The similarity between the CD4≤200 with and without TB is in agreement with the study by Dowdy et al., who found that participants with HIV alone had similar HRQOL as those with TB and HIV co-infection (20). The total score at baseline divided the groups in two, high CD4 count and low CD4 count groups. This showed that participants with lower CD4 counts had significantly decreased baseline total HRQOL scores when compared to the higher CD4 count groups, irrespective of TB, this was similar to the findings of Akimoro et al. (36). In addition, physical, emotional and functional wellbeing scores were also significantly different between the two groups (CD4>200 and CD4≤200), as would be expected. However, in the study by Deribew et al., the effect of CD4 count could not be determined (37).Dowdy et al. found that cross-sectional studies in TB-HIV co-infected patients had similar findings. Dowdy et al. found that physical domain was decreased in TB and HIV co-infected patients at start of treatment, while Venter et al. found that CD4 count had an effect on the physical domain (20, 38). In our study, the CD4 count was significantly lower in older patients, which we assumed was related to the length of time since the HIV diagnosis, which would be the result of the length of time since diagnosis and delay in starting ART(the government ARV programme had not started at this time). The difference in the experience of night sweats could not be explained.

For all scores combined, there was a highly significant change from baseline through month 12. Change in HRQOL scores over time showed that by the third month of treatment participants in all groups had improved significantly and at six months were almost similar, while at twelve months all participants had similar quality of life (39). This is consistent with other studies, Deribew et al. found that after six months

of treatment there was significant improvement in HRQOL in patients with TB and HIV and those with HIV alone (18).

Participants with CD4>200 improved the same whether they were on ARVs or not. As shown by Ralph et al., standard TB treatment was very successful in improving the HRQOL of patients with CD4 count > 200 not receiving ART(40). Those with CD4 count below 200 were started on ARVs within two weeks of starting anti-Tuberculosis treatment (ATT) and showed very quick response to treatment. The largest improvement was from baseline to month 3 in all groups, the lower the baseline CD4 count, the higher the rate of change observed. The experience of AEs was highest in the lower CD4 count group with TB followed by the non-TB lower CD4 count group. Low CD4 count and number of AEs were the main predictors of HRQOL. The study by Call et al. found similar significant differences in physical wellbeing between the CD4 count below and above 200 (34).

Almost all participants were smear-negative by end of two months and improvement in CD4 count was significant. The improvement could be the result of close monitoring that was done during the study. All patients had their own self-selected DOT supporter who was called before each visit to verify adherence. It was noticeable, however, that participants who took TB treatment only had slightly more SAEs at 6 months than others, however, by 12 months this had resolved. Four patients in the CD4>200 TB group were positive at month 5 or 6, while two patients in the CD4≤200TH group were positive during the same period. Decline in minimal important change in HRQOL was seen in patients who had decline in CD4 count and or had hepatic adverse events. According to Cella et al., patients can endure rather large negative changes in their QOL for the purpose of potential gains in the future (41).

The choice of timing and frequency of assessments was determined by the TB treatment outcomes, which are after the intensive phase of treatment at 2 months, and after treatment completion at 6 months. The HRQOL interview was conducted at month 3 to allow for all patients to complete the intensive phase. The next interview was at 6 months (TB treatment completion), and lastly at 12 months to evaluate full recovery. Despite lack of specific questions relating to TB specific symptoms, the FAHI questionnaire was able to effectively evaluate the HRQOL of TB and HIV co-infected patients. It was able to assess the differences between groups receiving combined TB and HIV therapy, from those receiving TB treatment only and non-TB group receiving ART only. When relating HRQOL to respiratory signs and symptoms, we found that

patients reporting respiratory signs and symptoms within the three months prior to the HRQOL evaluation, had lower scores at the next evaluation

The first limitation of the study was the small sample size as a sample of convenience was used. Also there were patients who did not have all the domains filled, and some patients withdrew before month 3. However, the observed changes in HRQOL are consistent with what would be expected for patients on treatment. When considering the number of AEs and SAEs over time, the results of the HRQOL make sense.

Another limitation of the study is that the questionnaire was not applied frequently enough. Although we were able to find the differences between groups, it may have been better if we evaluated the HRQOL more frequently in the first two months. As the questions from the FAHI questionnaire are based on the past seven days, there is the possibility that adverse effects could have been underreported. In addition, the CNS questionnaire was formulated to evaluate the side effects from Efavirenz, since these are more prominent during the first couple of weeks of starting treatment, the effects of Efavirenz may have been missed by this questionnaire. The score obtained for the cognitive domain, implies that the items of this domain did not form a good overall score. Also, during the three months preceding the application of the questionnaire, the participant may have had and resolved an adverse event before the past seven days.

In spite of the limitations listed, the data collected was able to show the differences between groups and was consistent with other findings in similar cohorts. Minimal important changes in HRQOL showing improvement in groups, as well as those showing worsening of condition, were related to the number of AEs and SAEs experienced in each group.

Conclusion

In this study we found that evaluating HRQOL in TB-HIV co-infected patients is useful as it gives the patients' subjective report on their treatment response. We found that respiratory signs and symptoms had an effect on HRQOL even after cure of TB (6 months), this means patients may need continued care even after completion of treatment. Evaluating HRQOL adds value to the monitoring of TB/HIV outcomes and would be helpful in evaluating patients that need continued support, even after cure.

Authors' contributions

TM participated in design, managed the study and drafted the manuscript. RR, KD and AP participated in the design and management of the study, TR performed the statistical analysis, SM helped draft the manuscript and assisted with the its final edit. All authors read and approved the manuscript.

Compliance with Ethical Standards

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Conflict of interests

There were no competing interests declared by any of the authors.

Ethical Approval:

The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (KZN), reference numbers E294/05 and BFC 037/08. All procedures performed in the study were in accordance with ethical standards of the national and institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Chapter 6

Urine lipoarabinomannan (LAM) and antimicrobial usage in seriously ill HIV-infected patients with sputum smear-negative pulmonary tuberculosis

Diagnosis of TB is commonly made when the patient presents with signs and symptoms of TB. The most common diagnostic algorithm involves testing of sputum for mycobacterium TB using smear microscopy or Gene Xpert MTB/RIF and sputum culture. In smear-negative TB or sputum scarce patients, such as those with HIV, diagnosis using a biological fluid either than sputum is needed. Urine is an easy to get fluid and a urine LAM test has been investigated in these populations. We investigated the usefulness of urine LAM to diagnose TB in seriously-ill smear-negative patients.

Thesis Objective 4 - To determine the utility of urine LAM in diagnosis of TB in seriously-ill HIV-infected patients with TB.

Sub-objective 4.1- To determine the utility of urine LAM for the diagnosis of smear-negative TB.

Sub-objective 4.2- To evaluate the potential utility of urine LAM to reduce antibiotic usage.

The manuscript below reported on urine LAM and antimicrobial usage in seriously-ill HIV-infected with smear-negative TB, addressing the above objectives.

Abstract

Background: Based on current WHO guidelines, hospitalized tuberculosis (TB) and HIV co-infected patients with CD4 count <100 cells/mm3 who are urine lipoarabinomannan (LAM) positive should be initiated on TB treatment. This recommendation is conditional, and data are limited in sputum smear-negative patients from TB endemic countries where the LAM test is largely inaccessible. Other potential benefits of LAM, including reduction in antibiotic usage have, hitherto, not been explored.

Methods: We consecutively enrolled newly-admitted seriously-ill HIV-infected patients (n= 187) with suspected TB from three hospitals in KwaZulu-Natal, South Africa. All patients were empirically treated for TB as per the WHO 2007 smear-negative TB algorithm (patients untreated for TB were not recruited). Bio-banked urine, donated prior to anti-TB treatment, was tested for TB-infection using a commercially available LAM-ELISA test. TB sputum and blood cultures were performed.

Results: Data from 156 patients containing CD4 count, urine-LAM, sputum- and blood-culture results were analysed. Mean age was 37 years, median CD4-count was 75 cells/mm3 (IQR [34–169 cells/mm3]), 54/156 (34.6%) were sputum culture-positive, 12/54 (22.2%) blood-culture positive, and 53/156 (34.6%) LAM-positive. Thus, LAM sensitivity was 55.6% (30/54. The study design did not allow for calculation of specificity. Urine-LAM positivity was associated with low CD4 count (p=0.002). Ninety percent (48/53) of LAM-positive patients received antibiotics [15/48 (31.3%), 23/48 (47.9%) and 10/48 (20.8%) received one, two or three different antibiotics respectively], while the duration of antibiotic therapy was more than 5 days in 26 of 46 (56.5%) patients.

Conclusion: Urine LAM testing in sputum smear-negative severely-ill hospitalized patients with TB-HIV co-infection and advanced immunosuppression, offered an immediate rule-in diagnosis in one-third of empirically treated patients. Moreover, LAM, by providing a rapid alternative diagnosis, could potentially reduce antibiotic overusage in such patients thereby reducing health-care costs and facilitating antibiotic stewardship.

KEYWORDS: tuberculosis, sputum, smear-negative, HIV, lipoarabinomannan

Introduction

The urine lipoarabinomannan (LAM) test has been shown not to be useful in untargeted patients with suspected tuberculosis (TB) in a primary health care setting (1), but it is useful for the diagnosis of hospitalized HIV-infected TB patients with a CD4 count <200 cells/mm^3, who would otherwise have required further investigation. This is a test based on detection of mycobacterial LAM antigen, a polysaccharide present in mycobacterial cell walls, which is excreted in the urine (2), now available as a point of care urine strip test. Given that between 24% and 62% of HIV-infected patients with TB present with sputum smear-negative findings (3), and that Xpert *Mycobacterium tuberculosis* (MTB)/ rifampicin (RIF) has limited availability in many countries, TB in patients living with advanced stage HIV is often difficult to diagnose. This category of patients frequently have difficulty in producing sputum (4, 5), and when combined with low sensitivity of sputum smear microscopy (6, 7), leads to delays in diagnosis (8) and treatment. Sputum microbiological culture is the only other definitive tool for the diagnosis of active TB (9), but it is labour intensive and the time to result is prolonged. The difficulty in the diagnosis of sputum smear-negative TB adds to the causes of failure to halt transmission and control the epidemic, especially in high burden countries. Diagnosis is therefore often made on clinical and radiological grounds, which is often atypical (10). Therefore, there is a need for tests that do not require sputum and are more sensitive to ensure early diagnosis.

Urine LAM has been investigated with varying results, but more so in sputum smear-positive patients (1, 11-32). There are few studies that have investigated the usefulness of urine LAM in diagnosing sputum smear-negative TB (33-35), but none have investigated the use of LAM in reducing antibiotic use. The use of antibiotics to treat suspected TB while waiting for confirmation of diagnosis in hospital is common, although in HIV-infected patients a response to antibiotics may not exclude TB (36). Considering the need to prevent antibiotics resistance, optimal and prudent use of antibiotics is necessary in line with antibiotic stewardship (37, 38). In this study, we investigated the diagnostic performance of LAM-ELISA in seriously-ill hospitalized HIV-infected patients with sputum smear-negative pulmonary TB (PTB) and its potential impact on antibiotic usage. The LAM-ELISA, since completion of this study, has been superseded by the LAM lateral flow assay but equivalence between the assays has been demonstrated (39, 40).

Methods:

Design and setting

This sub-study was conducted as part of a prospective cohort study evaluating the use of a WHO-recommended algorithm to reduce mortality in seriously-ill HIV-infected patients with sputum smear-negative PTB in South Africa (36). Participants were recruited from three hospitals around Durban between March and December 2009.

Participants

Patients with advanced immune suppression and symptoms of TB requiring hospitalization, and with smear-negative sputum, were consecutively enrolled once informed consent was obtained. At one time point on admission, before commencement of antibiotics or anti-TB therapy (ATT), the following samples were collected from consenting participants: spot expectorated sputum and blood for culture, and midstream urine for LAM test. Participants were excluded if they were 1) currently being treated for latent TB infection (LTBI); 2) treated for TB disease; or 3) were found not to be HIV-infected based on lack of "strong clinical HIV evidence" or an HIV test at either screening, admission or during hospitalization. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (KZN) and an Institutional Review Board at the U.S. Centers for Disease Control and Prevention (CDC).

Patients were started on TB treatment based on criteria defined in the parent study (36): 1) had at least 2 negative specimens for acid fast bacilli; 2) radiographic abnormalities consistent with active TB (according to the treating medical practitioner); 3) laboratory evidence of HIV-infected or strong clinical evidence of HIV infection, later confirmed by a laboratory test and 4) a decision by a clinician to treat with a full course of anti-TB treatment (ATT) or sputum smear-negative for acid fast bacilli but culture positive for *Mycobacterium tuberculosis* (MTB).

Sputum and blood processing

All sputum samples were processed using NALC/NaOH and microscopy smear using both Auramine and Ziehl-Neelsen staining and were cultured on Middlebrook 7H11 selective agar (Difco) and MGIT liquid medium (Becton Dickenson, Sparks, USA). Positive *Mtb* cultures were confirmed with niacin and nitrate biochemical testing. All blood samples were collected by venipuncture and cultured into aearobic and anaerobic culture systems performed on BacT/ALERT MB (bioMerieux, Marcy-l'Etoile, France) systems.

Urine LAM-ELISA

Urine samples were processed according to the manufacturer's instructions (Clearview TB® ELISA, Inverness Medical Innovations, Inc., Waltham, U.S.A.); within 24 hours of collection urines were boiled at 95–100°C for 30 minutes, centrifuged for 15 min at 10000 rpm and aliquots of 1 ml of the supernatant stored at -70ºC. The assay was performed on the stored aliquots once a week.

From each frozen aliquot, 100 µl sample was incubated for 60 min at ambient temperature, and washed with Phosphate Buffered Saline pH 7.4/Tween-20 (PBST). Subsequently, 100 µl of undiluted conjugate solution (HRP-conjugated LAM-specific rabbit polyclonal antibody) was added. After a 60 min incubation and washing with PBST, 100 µl of the colour developer tetramethylbenzidine (TMB) was administered to each well. The substrate was again incubated for 15 min at ambient temperature, and the reaction was stopped by adding 100 µl of stop solution (1 M H_2SO_4). The colour development was measured at 450 nm on an automated ELISA plate reader. Samples and negative and positive controls were run in duplicate.

Prior to interpretation of the urine LAM-ELISA results, the validity of each run was confirmed as per the manufacturer's package insert as: i) negative control mean optical density (OD) was >0.1 OD and <0.3 OD and ii) positive control mean OD was >0.3 and ≤0.5 above the negative control. Differences for each sample between the two runs were examined to see if their difference were not >15.0%. If the mean OD of the sample aliquots were ≥0.1 above the mean OD of the negative controls, the sample was considered urine LAM-positive. LAM-ELISA and the LAM strip test have been found not to be significantly different in studies comparing these two versions (39, 40).

Chest radiograph

Chest radiographs (X-rays) were initially read by the treating medical practitioner and then sent to an independent radiologist for reading. The results were then sent to the hospital concerned for the benefit of the medical practitioner. The external radiology results did not directly affect the start of treatment.

Statistical methods

Sample size was not calculated for this sub-study but we used the second cohort of the main study (36). Descriptive statistics were used to summarize the data. Comparisons of categorical data between subgroups were made using Chi square tests or Fisher's exact tests as appropriate. Numeric variables

such as CD4 and time to event were compared using Mann-Whitney "U" test, while t-test was used for age. Standard measures of diagnostic efficacy are reported where culture is considered the gold standard. Data were entered in Epidata and analysed in Stata V13.1. Kaplan Meier was used to analyse time to death or discharge and a log rank test used to compare subgroups. Median time to death or discharge were compared using Mann-Whitney tests. Statistical analysis were performed using Stata 13 and a $p \leq 0.05$ was considered significant.

Results:

Demographic characteristics

We enrolled a total of 187 patients, (Figure 17), out of which 168 had sputum and urine LAM-ELISA results and 156 had all results including CD4 count and were analysed. Table 18 shows the demographic characteristics; 87 (55.8%) patients were male and 154 (98.7%) were black, 81 in WHO stage 3 (51.9%) and 75 (48.1%) in WHO stage 4 (41). Twenty-four (15.4%) were on ART and 43 (27.6%) had a history of prior TB treatment. Median CD4 count was 75 cells/mm3, IQR (34.5–169). 50/156 (32.1%) of patients had a CD4 count <50 cells/mm^3; 42/156 (26.9%) had CD4 count between 50 cells/mm^3 and 100 cells/mm^3; 35/156 (22.4%) had CD4 count between 100 and 200 cells/mm^3; and 29/156 (18.6%) had CD4 count ≥200 cells/mm^3.

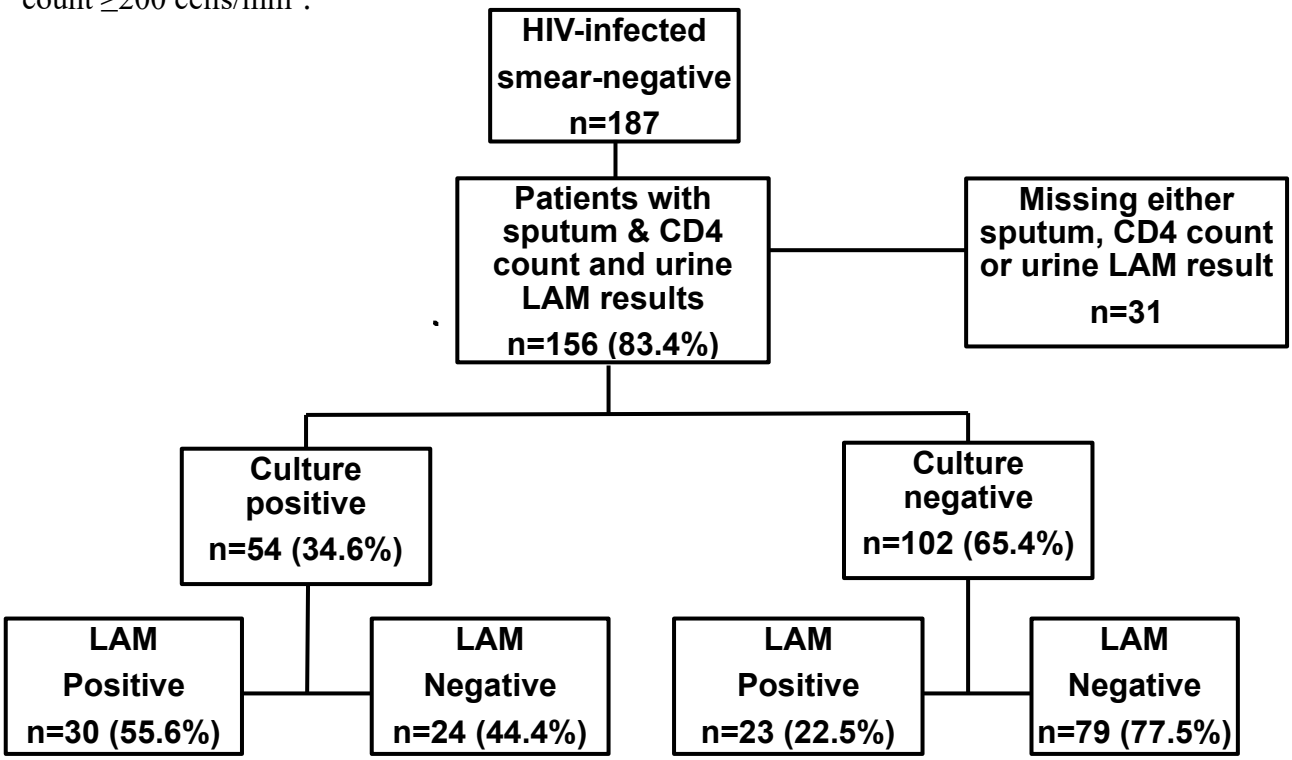

Figure 17: Participant flow diagram of seriously-ill HIV-infected hospitalized patients with sputum smear-negative pulmonary tuberculosis.

Diagnostic accuracy

Out of the 156 patients analysed, 54/156 were sputum culture-positive (34.6%), 53/156 (34.0%) were urine LAM-positive. Thirty urine LAM-positive patients were culture-positive, therefore, the overall test sensitivity was 55.6%. Of the 30 urine LAM-positive/culture positive patients, 21 70.0%) had CD4 count <100 cells/mm3. Urine LAM sensitivity for patients with CD4 count <100 cells/mm^3 was 63.6% (21/33). Urine LAM sensitivity for patients with CD4 count 100–200 cells/mm^3 was 57.1% (9/14) and among those with CD4 count >200 cells/mm^3, it was 14.3% (1/7). Among the culture negative patients, 23 (22.5%) were urine LAM-positive, as seen in Figure 18.

Sputum, chest radiograph, urine LAM and blood culture

Figure 18 shows culture positivity, with 54/156 (34.6%) sputum culture positive patients with at least one sputum sample. Although all patients' diagnosis included chest radiography, only 120/156 (76.9%) patients had their chest X-rays read by an independent radiologist. Of the 53 urine LAM-positive patients 35/53 (66.0%) had a chest X-ray read by the radiologist and 20/35 (57.1%) were consistent with PTB. The majority, 52/53 (98.1%), of the urine LAM-positive patients had blood culture ordered, 11/52 (21.2%) of whom were sputum and blood culture positive and 32/52 (61.5%) blood culture negative, with 7/52 (13.5%) contaminated and 2/52 (3.8%) not done. Only 1/54 (1.8%) culture positive patient was urine LAM-negative and 8/11 (72.7%) blood culture positive patients had a CD4 ≤100 cells/mm^3.

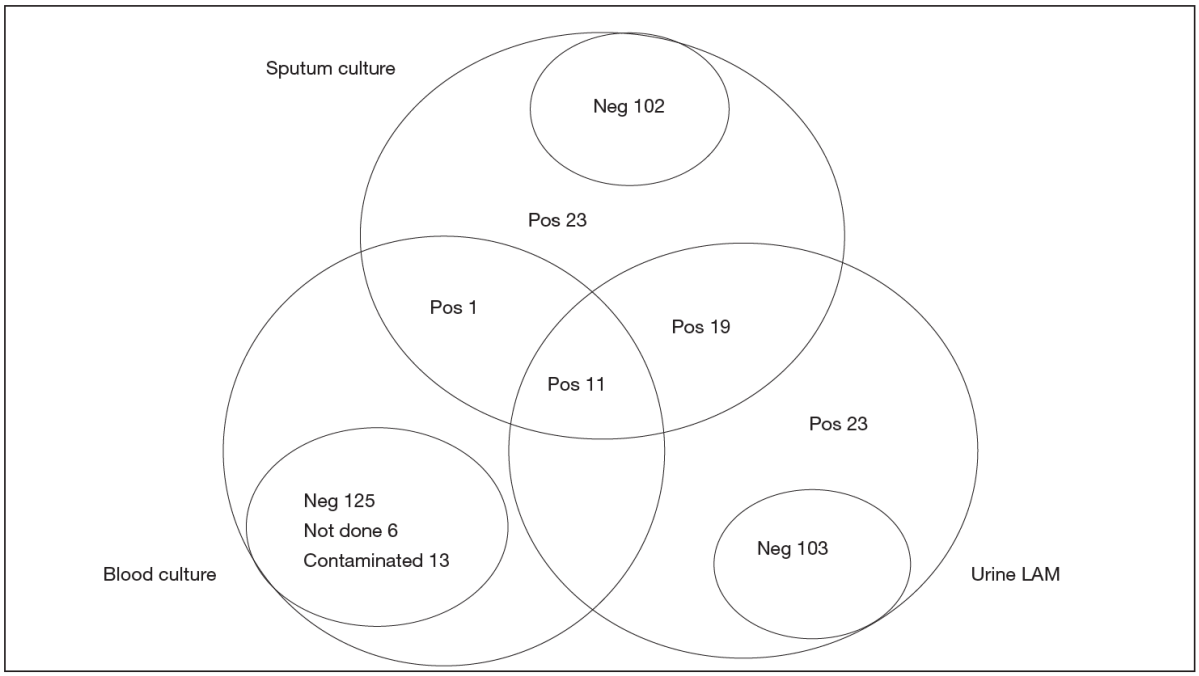

Figure 18: LAM, sputum and blood culture, in seriously-ill, HIV-infected patients with sputum smear-negative pulmonary tuberculosis (n=156)

As shown in Table 20, of the 12 blood-culture positive patients, 6 (50.0%) were confirmed as not PTB by the radiologist.

Table 18: Demographic characteristics of seriously-ill HIV-infected patients (n=156)

	Culture & LAM											
	Total		Pos				Neg				p value	
	n=156		Culture: n=54		LAM: n=53		Culture: n=102		LAM: n=103		Culture	LAM
Characteristics	N	%	N	%	N	%	N	%	N	%		
Sex												
Male	87	55.8%	36	66.7%	34	64.2%	51	50.0%	53	51.5%	0.04646	0.131
Female	69	44.2%	18	33.3%	19	35.8%	51	50.0%	50	48.5%	.	
Race												
Black	154	98.7%	54	100%	53	100%	100	98.0%	101	98.0%	1.000†	1.000†
Indian	1	0.6%	0	0%	0	0%	1	1.0%	1	1.0%		
White	1	0.6%	0	0%	0	0%	1	1.0%	1	1.0%		
Clinical Stage												
Stage 3	81	51.9%	32	59.3%	28	52.8%	49	48.0%	53	51.5%	0.182	0.873
Stage 4	75	48.1%	22	40.7%	25	47.2%	53	52.0%	50	48.5%		
On ARV												
Yes	24	15.4%	7	13.0%	7	13.2%	17	16.7%	17	16.5%	0.542	0.589
No	132	84.6%	47	87.0%	46	86.8%	85	83.3%	86	83.5%		
Prior ATT												
Yes	43	27.6%	9	16.7%	8	15.1%	34	33.3%	35	34.0%	0.027	0.012
No	113	72.4%	45	83.3%	45	84.9%	68	66.7%	68	66.0%		
CD4 group												
< 50	50	32.1%	15	27.8%	18	34.0%	35	34.3%	32	31.1%	0.310†	0.016†
50-<100	42	26.9%	18	33.3%	18	34.0%	24	23.5%	24	23.3%		
100-200	35	22.4%	14	25.9%	14	26.4%	21	20.6%	21	20.4%		
> 200	29	18.6%	7	13.0%	3	5.6%	22	21.6%	26	25.2%		
X-ray												
Not TB	38	24.4%	17	31.5%	15	28.3%	21	20.6%	23	22.3%		
TB	57	36.5%	19	35.2%	18	33.9%	38	37.3%	39	37.9%		
Atypical TB	7	4.5%	1	1.9%	1	1.9%	6	5.9%	6	5.8%		
Pleural effusion	1	0.6%	1	1.9%	1	1.9%	0	0%	0	0%		
signed out	1	0.6%	0	0%	1	1.9%	1	1%	0	0%		
Transfer	3	1.9%	2	3.7%	2	3.8%	1	1%	1	1.0%		
Missing	49	31.4%	14	25.9%	15	28.3%	35	34.3%	34	33.0%		
Anti TB treatment started												
0-5 days	121	77.6%	44	81.5%	42	79.3%	77	75.5%	79	76.7%	0.393	0.718
6-22 days	35	22.4%	10	18.5%	11	20.7%	25	24.5%	24	23.3%		

†, comparison done using fisher's exact test. LAM, lipoarabinomannan; ART, antiretroviral therapy; ATT, anti-TB therapy; TB, tuberculosis.

Factors associated with a positive urine LAM result

Prior ATT was associated with a negative urine LAM result at the univariate level, 8 (15.1%) of urine-LAM-positive patients had prior ATT compared to 35 (34.0%) of urine-LAM-negative patients (OR: 2.6; 95%CI: 1.2-5.9, p=0.02.). A positive urine LAM result was also associated with a CD4 count <200, (50/127) 39.4%; compared to 10.3% (3/29) patients with CD4 count >200 (OR: 5.6; 95%CI: 1.6-19.6, p = 0.007. In a multivariable model containing variables significant at the univariate level, both remained independently associated with a positive LAM result: (OR_{adj}:3.2; 95%CI: 1.3-7.6, p=0.009). (OR_{adj}:6.2; 95%CI: 1.821.8, p=0.005) respectively.

Antibiotic use and ATT

The majority 48/53 (90.6%) of the urine LAM-positive patients received antibiotics and 18/46 (39.1%) with recorded time, were on antibiotics for a median time of 5 days (Table 19). Of the 48/53 (90.6%) LAM -positive patients that received antibiotics, 10 (20.8%) received three types or more while 23/48 (47.9%) received two types and 15/48 (31.3%) received one type of antibiotics. All patients received ATT, of the 53 urine LAM-positive patients, 32 (60.4%) received ATT within 3 days of admission as per protocol, and the rest within 4–22 days.

Table 19 Potential benefits of early diagnosis with LAM in seriously-ill HIV-infected patients with sputum smear-negative pulmonary tuberculosis (n=53)

Treatment and length of stay	LAM positive (n=53)
Antibiotics given, n (%)	
Yes	48 (90.6)
No	5 (9.4)
Time on antibiotics (n=46, 2 patients missing time), median [IQR] (days)	5 [3–7]
Time on antibiotics (n=46), n (%)	26 (56.5)
<5 days	20 (43.5)
≥5 days	
Number of antibiotics (n=48), n (%)	
1	15 (31.3)
2	23 (47.9)
3 or more	10 (20.8)
Start of TB treatment (n=53), median time of start of TB treatment, median [IQR] (days)	3 [2–4]
0–3 days, n (%)	33 (63.3)
4–22 days, n (%)	20 (37.7)
Length of stay (n=53), n (%)	
0–3 days	12 (22.6)
4–6 days	25 (47.2)
7 days and above	16 (30.2)

Treatment outcomes - time to discharge and time to death

Median time to discharge, excluding deaths was 5 days, (IQR 4–6) for the urine LAM-positive (n=48) and 6 days (IQR 4–8) for the urine LAM-negatives (n=99), and was statistically significantly different, (p=0.03). Median time to death was 12 days (IQR 11–13) for the urine LAM-positive (n=5) and 7 days (IQR 7–8), for the urine LAM-negative (n=4) but was not significantly different (p=0.11).

Discussion:

We evaluated the use of urine LAM for the diagnosis of smear-negative TB and found that more than 52% of culture-positive patients were urine LAM-positive. Moreover, nearly 20% of sputum smear- and culture-negative presumptive TB patients were urine LAM-positive.

Almost all the patients in this study received empiric antibiotics. With the availability of LAM results, in at least one third of the patients, antibiotic usage could have been obviated or curtailed. Although all patients in this study should have been started on ATT within 3 days, (as per 2007 WHO guidelines for treatment of smear-negative TB and study protocol), and all were found to have chest X-ray abnormality consistent with TB by their treating clinician, we found that at least 10 LAM-positive patients were given three or more types of antibiotics with or without ATT for more than 5 days (42.2%). This was probably to cover the patients for other bacterial infections should TB not be confirmed, although there were no confirmed bacterial infection tested on these patients. In addition, nearly 40% of the 53 urine-LAM-positive patients received ATT within 4-22 days, and the delay may have been due to the need to allow antibiotics time to act while waiting for sputum-culture results.

It is reasonable to assume that fewer antibiotics could have been used had the urine LAM results been available on the day of admission. Peter *et al* suggested that the urine LAM assay may be used as a "rule in" test for smear-negative HIV-infected patients (42). Using LAM as a rule in test, in conjunction with clinical signs and symptoms and radiographic findings, urine LAM-positive patients could receive ATT within hours of admission, saving excessive use of antibiotics. This would be in line with current country-specific and antibiotic stewardship guidelines. Indeed, Padayatchi *et al* have advocated for ensuring that patients receive appropriate treatment with specific attention to dosing and duration as the first goal of antibiotic stewardship preventing drug overuse and abuse as the second goal, and minimizing TB drug resistance at an individual and community level as the third goal (43). However, clinicians in many settings still rely

strongly on antibiotic trials for smear-negative TB (44). There are recent studies investigating the use of trial of antibiotics as a diagnostic tool for smear-negative TB (45) indicating that the practice is ongoing. A study by Walusimbi *et al* (46) recommends that it is useful to start HIV-infected smear-negative patients on an initial course of antibiotics and in South Africa, four of the five patients given antibiotics while waiting for culture results, (patients were smear and Xpert negative) could not be started on ATT as they were lost to follow up by the time the results were available, and recommended to start HIV-infected sputum smear-negative patients on an initial course of antibiotics (47). In this setting, a positive urine LAM test is likely to reduce antibiotic usage.

Table 20: Seriously-ill HIV-infected patients with sputum smear-negative pulmonary tuberculosis and with positive blood culture (n=12)

PID	History of TB Yes[1]/No[0]	Radiology X-ray reading	Sputum culture result positive	Blood culture result positive	Urine LAM-positive	CD4 value cells/mm^3	Dead/Alive after 56 days	Days to positivity
1334	0	Not TB	1	1	1	97	1	6
1376	0	TB	1	1	1	63.1	1	5
1543	0	Not TB	1	1	0	79.4	1	17
2466	0	Not TB	1	1	1	88	1	8
3247	0	Not TB	1	1	1	65	1	24
3284	0	NA	1	1	1	26	0	NA
3317	0	NA	1	1	1	74	1	NA
3372	0	TB	1	1	1	68	0	8
3404	0	NA	1	1	1	106	1	9
3406	1	Not TB	1	1	1	68	1	9
3434	0	Not TB	1	1	1	128	1	11
3508	0	NA	1	1	1	176	0	31

Yes = 1 and No = 0; Positive = 1; Alive = 1; Dead = 0; Not available = NA

We found that a positive blood culture was one of the factors associated with a positive urine LAM, consistent with findings of Shah and colleagues (20). More than 80% of patients with positive blood culture were positive for urine LAM, and 58% of them had chest X-rays not consistent with TB by a radiologist (Table 19). Thus, urine LAM, being of much lower cost and a point of care assay, could replace blood culture as a screening investigation in HIV-infected hospitalised patients with fever and advanced HIV disease.

Our study had several limitations. We did not enroll a control group, and since all the patients were either culture-confirmed TB or clinically diagnosed TB, we could not calculate the specificity. A limited autopsy study by Cohen et al found that almost half of the HIV co-infected inpatients who died had evidence of

TB at the time of death (48). More than half were not on TB treatment, and based on this information from the same geographical area as our study, we could not confirm that the culture negative patients did not have TB disease. The LAM-ELISA, since completion of this study, has been superseded by the LAM lateral flow assay but equivalence between the assays has been demonstrated (18, 39). Fujifilm SILVAMP TB LAM assay was assessed in comparison to AlereLAM by Broger et al, and the findings were that FujiLAM offers superior diagnostic sensitivity, while maintaining specificity, and has the potential to transform rapid point-of-care tuberculosis diagnosis for hospitalized PLHIV (49).

We did not directly demonstrate a reduction in antibiotic usage though it is likely this could have been substantially curtailed in urine LAM-positive patients. This will need to be evaluated in a future prospective study using LAM and remains an important question to be answered.

In summary, urine LAM testing in sputum smear-negative severely ill HIV-infected hospitalized patients with suspected TB and advanced immunosuppression offers the potential to start ATT early and to decrease the number and duration of antibiotics used.

Contributors

TM, JA, THH and RR contributed to the study conception and designed the study. TM and JA, contributed to acquisition of data. CC, LM, KD and TM analysed and interpreted the data and provided statistical expertise. TM and KD drafted the report; and RR, JA, JP, MD, AP and LM contributed to revision of the report. JA coordinated laboratory support and contributed to the revision of the report.

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Conflicts of interest

We declare that we have no conflict of interest.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the funding agencies.

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Chapter 7

Discussion and conclusions

In this thesis we report four different types of biomarkers. We evaluated the relationship of NAT2 acetylation genotype and phenotype, QFT-GIT's usefulness in monitoring TB-HIV treatment response, HRQOL for monitoring of TB-HIV treatment using the subjective view from patients, and urine LAM for diagnosis of smear-negative TB.

The first analysis, reported in Chapter 3 of this thesis investigated the *NAT2* genotype of predominantly IsiZulu speaking black South Africans from Durban and surrounding areas. We used six of the seven most frequent SNPs (NAT2 nomenclature) in the human population. Suarez-Kurtz et al found 100% agreement between the inferred acetylator phenotypes using either seven- or four-SNP genotype panels in six study groups including admixed populations (1). Their study included blacks, although these were not South Africans. We found that: 1) the slow acetylator genotype was the most prevalent, (52.5%), compared to intermediate (35.8%) and rapid (11.7%). 2). There was a high prevalence of *NAT2*5* and *NAT2*6* in our population, contributing to the slow acetylator genotype. The pharmacokinetics analysis conducted in a subset of participants, study 2, showed an increased variability INH pharmacokinetics and its metabolite, AcINH, but was not statistically different. There was a high level of discordance between the deduced phenotype, and actual phenotype (derived from the ratio of the 3-hour and 6-hour concentrations of the mean log Ac-INH/mean log INH), and the molar (metabolic) ratio, derived from parent drug over the metabolite, from the PK results.

We reported the effects of *NAT2* genetic polymorphisms on the pharmacokinetics of isoniazid and its effect on hepatotoxicity in two studies. Research has shown that response to treatment is not uniform, with some people responding better than others (2, 3). Research has also shown that there are people who are genetically more prone to certain diseases than others (4). When evaluating treatment experiences we should also understand the influence of genetic interactions involved.

There have been conflicting reports on whether slow or rapid acetylators are more prone to hepatotoxicity. Most research reports indicate that slow acetylators are more likely to present with hepatotoxicity (5, 6).

Our analysis did not show conclusively which acetylator phenotype was more prone to hepatotoxicity. We found that the genetic and phenotypic picture was discordant at most.

Genetic studies have been used to determine similarities and difference between individuals. Different population groups have been found to have certain predisposition to certain conditions that other race groups don't have. We report the findings on NAT2 polymorphisms among Zulu speaking black South Africans, which differ from other black South Africans. We found that although NAT2 genotype indicated higher frequencies of slow and intermediate acetylator genotypes, isoniazid PK suggested a higher frequency of rapid acetylator phenotypes. This was different from other findings in HIV-infected individuals which suggest a tendency to slow phenotype. There was a diversity of specific *NAT2* alleles of a pattern differing from previously studied cohorts in other settings. Investigation of the contribution of other factors including further genetic variants, in this population, is warranted, this was beyond the scope of this project.

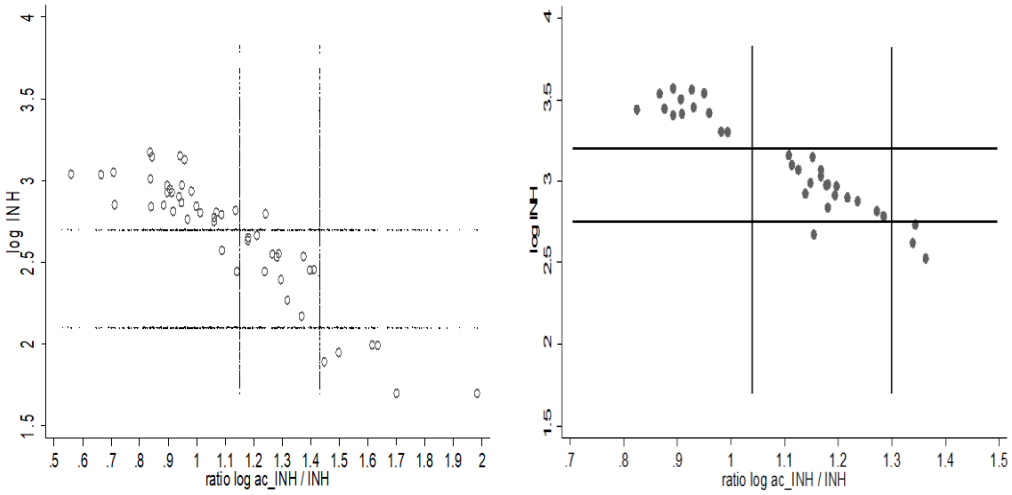
One disturbing finding was the low concentration of INH, with C_{max} below the lower limit of target range in 98.3% patients in study 1 and 47.5% patients in study 2 because multidrug resistant TB is high in the region of the studies and this may contribute to its prevalence. Importantly *NAT2* genotype did not explain PK variability in this cohort or the low C_{max}, which suggests that other factors could be influencing isoniazid bioavailability and metabolism, which require further elucidation.Another study conducted in the same population found reduced isoniazid levels (7). There may be value in re-evaluating the dose ranges for patients in this population.

Adverse events to isoniazid, such as hepatotoxicity and peripheral neuropathy are expected to be higher in slow acetylators. In our study we found no peripheral neuropathy, all patients were on pyridoxine, which may have prevented the peripheral neuropathy. Only one patient, who was a rapid acetylator, had grade 4 hepatotoxicity and the rest had grade 1 and 2 elevation in liver enzymes. It is possible that the dose range was responsible for the reduction in liver toxicity, which was less than the expected 2 – 28% (8). All our patients were less than 55 years old, this could have contributed to the low rate of hepatotoxicity.

The phenotype determination using the pharmacokinetics of isoniazid was not comparable to the genotype, with a high rate of discordance noted. Since most intermediate participants carried a 12A, B or

C in the haplotype, there is a possibility of misclassification as detailed by Agundez et al. in their study (9). They indicated that an individual with a slow haplotype in combination 12A, B, C, D and 13, may be misclassified as slow. For our interpretation, our participants with this combination were classified as intermediate, but it is possible that that the rapid allele was more dominant. Based on this assumption, the behaviour of the pharmacokinetics parameters, leaning more towards rapid than slow could be influenced by this misclassification. In this group, *NAT2**5 occurred in combination with *12A or *12C. While this might explain the intermediate acetylator genotypes mimicking the rapid acetylators, it does not explain the slow genotypes that were all *5/*5 showing rapid acetylation profile. The only participant with rapid genotype in this group was homozygous *12C/*12C. This participant was the only participant with drug induced hepatitis in this group. The rest of the adverse events in this group were almost evenly distributed between the slow and the intermediate groups.

When predicting phenotype, there was no clear differentiation at two hours. Using a 4-hour post-dose cut-off time for the acINH:INH ratio (10), the separation between slow and intermediate/rapid acetylators was not obvious for study 1, while for study 2, a clear separation of the acetylator phenotypes was apparent. Using PK analysis alone, there were 6% rapid, 27% intermediate and 60% slow phenotypes in study 1, and 9% rapid, 52% intermediate and 39% slow in study 2, respectively, which is in agreement with the NAT2 data.



Study 1 Study 2

Using different phenotype classification methods, there was notable discordance between genotype and phenotype, irrespective of the method. The percent slow was similar for the metabolic ratio or plasma

concentration ratio of AcINH/INH and the molar ratio of INH/AcINH but the intermediate and rapid phenotypes were very different. The distribution of adverse events experienced did not follow the trend as both the slow and intermediate genotype had similar rates of adverse events. Although there were increased liver enzymes, only one participant, who happened to be rapid genotype experienced grade 4 drug induced hepatotoxicity. There was no difference in the distribution of grade 1 and 2 liver enzymes among the groups.

The study limitations included the small sample size and the lack of an HIV-uninfected comparator group.

In chapter 4 we reported on the kinetics of IGRA. QFT-GIT test was conducted at baseline, month three, month six of ATT and at month twelve, six months after completion of ATT. QFT-GIT readouts generally decreased with time during anti-TB treatment, irrespective of whether patients were on ART. We also found that IGRA responses did not correlate with 2 or 6-month culture-conversion and markers of bacillary burden. ART alone (without active TB) resulted in a significant increase in IGRA responses by month 3. This is likely due to ART-related immune reconstitution and redistribution of effector memory T-cells back into the blood compartment(11). Similarly, ART can cause apparent conversion of the TST whereby TST negative persons become TST positive on ART (12, 13). These data have important implications for interpretation of immunodiagnostic tests in the context of TPT provision in high burden settings as current data suggest that only TST positive persons are likely to benefit from TPT (14, 15).

Very few studies were conducted in TB and HIV co-infected patients to evaluate QFT-GIT usefulness in monitoring treatment and all agree that it cannot be used. Based on this, the WHO has issued guidelines that IGRAs should not be used to diagnose TB especially in high burden areas (16). In low burden areas IGRAS have been used successfully to diagnose latent TB. In this study participants continued to have positive QFT-GIT six months after completion of ATT, although there were no signs and symptoms of relapse or reinfection. The latest test, QFT-Plus showed similar sensitivity to QFT-GIT but there are no studies that have evaluated it longitudinally in active TB patients. Had the QFT test worked to diagnose and monitor TB, the cost would be unaffordable for most TB programs.

Limitations of this study include the small sample size and the use of QFT-GIT, which is not the most suitable test for measuring IFN-γ. We found no correlation between bacterial load and IGRA. The lack of

reversion of IGRA in most patients support the findings that this test should not be used to monitor treatment and is in line with findings of other researchers.

Chapter 5 reported on HRQOL for monitoring TB-HIV therapy. When evaluating HRQOL, we found that HRQOL is useful in assessing tolerability and safety of TB-HIV treatment in co-infected patients. The improvement in HRQOL was not related to the treatment type as patients improved the same whether they were on ATT alone or with ART. This is important as participants taking ATT only were those with CD4 count above 200 cells/mm^3. HRQOL at baseline was associated with baseline CD4 count, and the number and type of adverse events experienced by participants during follow up was associated with reduced HRQOL. It was expected that participants with CD4 count below 200 cells/mm^3 would be worse off than those with higher CD4 counts, as has been reported in other studies (17). The disease progression for these participants would also contribute to the decline in HRQOL. Although we found that CD4 count was the main risk factor for reduction in HRQOL, the lower the CD4 count the lower the HRQOL scores, it was this group that showed the most improvement is HRQOL scores. This is encouraging as in our setting we found that most people delay testing and thereby delaying in start of ART. In South Africa all TB patients are offered an HIV test and started on ART as soon as possible while on ATT, this is in line with the current WHO guidelines (18).

When evaluating meaningful decline in HRQOL we found that not taking ART with TB treatment is a risk factor for decline in CD4 count and HRQOL. This was relevant for the participants on the ATT only arm as the commencement of ART was delayed. At the time of the study, only people with CD4 count below 200 cells/mm^3 were eligible for ART. These findings are in line with current literature on this subject. The total score at baseline divided the groups in two, high CD4 count and low CD4 count groups. This showed that participants with lower CD4 counts had significantly decreased baseline total HRQOL scores when compared to the higher CD4 count groups, irrespective of TB, this was similar to the findings of Akimoro et al. (19). In addition, physical, emotional and functional wellbeing were also significantly different between the two groups (CD4>200 cells/mm^3 and CD4≤200 cells/mm^3), as would be expected. However, in the study by Deribew et al., the effect of CD4 count could not be determined (20). Dowdy et al. measured QOL among individuals receiving HIV therapy, active TB treatment and therapy for both TB and HIV. Since this was a cross sectional study, adverse effects of treatment were not analysed. They found that cross-sectional studies in TB-HIV co-infected patients had similar findings. They also found that physical domain was decreased in TB and HIV co-infected patients at start of treatment, while Venter

et al. found that CD4 count had an effect on the physical domain (21, 22). In our study, the CD4 count was significantly lower in older patients, which would be expected since they had not started ARVs by the time of the study. The addition of the 10 items on central nervous system to the HRQOL investigation did not add much value. The CNS domain behaved the same as the cognitive domain, with differences seen only between high and low CD4 count groups. The explanation for this would be that the application of the questionnaires was sparsely spaced. The efavirenz side effect are expected immediately after commencement of therapy and at most very few people experience these after two weeks of therapy (23, 24).

In order to be user friendly, an instrument should be of short to medium length of between 10 – 20 minutes as patients may not want to be held up in the clinic for long and the clinic staff may be overwhelmed with routine work. If the instrument is in local language, it can be handed to patients to fill as they are waiting to be seen by clinic staff. The clinic staff can then check it for completion and only help those patients who are unable to fill it themselves. An instrument with these features would help the TB program understand the subjective view of the patients' quality of life during combined TB and HIV treatment.

This study had the same limitations as the other two studies i.e. the small sample size and the lack of an HIV-uninfected comparator group.

The study reported in chapter 6 investigated the diagnostic performance of LAM-ELISA in seriously-ill hospitalized HIV-infected patients with sputum smear-negative pulmonary TB (PTB) and its potential impact on antibiotic usage. Urine LAM as a diagnostic biomarker in patients with smear-negative TB was investigated. We found that urine LAM was useful for the diagnosis of TB in seriously-ill smear-negative patients and in that it could potentially reduce antibiotic overuse. We used culture as a gold standard, but since all patients were diagnosed clinically and by chest X-ray, culture-/LAM+ patients, 23 of 53 (53%) urine LAM positive patients, is the group that urine LAM had added value (those who would not have been diagnosed by sputum culture).

The sensitivity was similar to other studies, with higher sensitivity in lower CD4 count groups. This was similar to a study by Dheda et al. (25, 26) and others, sensitivity was highest in patients with CD4 count below 100 cells/mm^3. Since this study, the LAM-ELISA, has been superseded by the LAM lateral flow assay (LF-LAM) (Alere), but equivalence between the assays has been demonstrated (27, 28). Urine LAM

has been included in the guidelines for diagnosis of TB in HIV co-infected patients with CD4 count less than 100 cells/mm^3 (29). The global TB report for 2019 has a statement on updating the 2015 guidelines to strengthen "indication for the use of LF-LAM among hospitalized HIV-positive patients with signs and symptoms of TB (pulmonary and extrapulmonary); the test is now recommended for all such patients, irrespective of their CD4 count. If the CD4 count is below 100, LF-LAM is recommended even in the absence of TB symptoms."(30).

The findings of our study support the use of urine LAM for diagnosis of TB in this population as it includes the HIV-infected smear-negative individuals, as indicated in Chapter 3. Additional investigations would still be needed for susceptibility testing to ensure that correct treatment is prescribed. Although the sensitivity was not high in sputum culture positive patients, urine LAM was positive for all blood culture positive patients. This finding is important in that TB treatment can be commenced. We also know that culture does not diagnose all pulmonary TB, either because of low bacterial load or poor sputum sample. Since LAM will diagnose some of these patients with culture negative TB, they can also be commenced on TB treatment early. This may potentially help reduce the number of antibiotics used in empiric treatment thereby improving antibiotic stewardship. The use of urine LAM in TB diagnosis is the step in the right direction, but the search for a better TB diagnostic continues.

The limitations of this study were that we did not extensively explore extra pulmonary TB findings. We also followed patients up after 8 weeks to ask about treatment outcomes but did not follow up to treatment completion. Although all patients received treatment for TB, we could not confirm whether they all continued TB treatment after discharge from hospital. Another limitation of the urine LAM test is its low sensitivity in patients with high CD4 counts, its failure to detect drug resistant-TB, and its failure to diagnose TB in HIV-uninfected patients.

We concluded that urine LAM testing in sputum smear-negative severely-ill hospitalized patients with TB-HIV co-infection and advanced immunosuppression, offered an immediate rule-in diagnosis in one-third of empirically treated patients. Also, urine LAM, by providing a rapid alternative diagnosis, could potentially reduce antibiotic over usage in such patients thereby reducing health-care costs and facilitating antibiotic stewardship.

Conclusions

With the current difficulties in diagnosis of all forms of TB and monitoring of its treatment response, it would be beneficial to find biomarkers that would fulfil this unmet need. Although the biomarkers discussed here have some limitations and benefits, we still have not found one biomarker optimal for both diagnosis and monitoring, the search continues. There were interesting results showing that genotype may not be a useful biomarker for prediction of treatment effects, including adverse events. The knowledge of acetylator genotype was not necessarily interchangeable with phenotype in this cohort. Until more studies show high levels of genotype-phenotype concordance, knowing the genotype would not help the TB program and therefore it is not recommended that it be used to plan treatment of TB-HIV co infected patients. In addition, this study did not suggest acetylator genotype had an influence in the experience of adverse events.

IGRAs do not appear to be useful for the prognostication of treatment outcome in co-infected patients on concurrent therapy. As already recommended by the WHO, the QFT-GIT test should not be used in monitoring TB-HIV therapy.

In addition to existing knowledge, this report brings important information to use when planning future studies. More work needs to be done in new diagnostics development as well as TB-HIV monitoring tools to cut down the time to diagnosis and to ensure rapid intervention in patients failing treatment. Also, the existing monitoring tools need improvement to ensure their availability at point of care. There is limited research available on HRQOL of TB and TB-HIV co infected patients in our country. Because South Africa is multi-lingual, multi-cultural country, the existing instruments need to be translated to local languages and validated or tested on local patients. It would be useful to ensure that more HRQOL instruments are made available and their use encouraged.

Our recommendations are that HRQOL would benefit the program if included as a tool to monitor effect of treatment. The choice of an instrument should consider shorter and disease specific instruments that can be easily included without taking too much of the clinic staff or patients' time. In order to be user friendly, an instrument should be of short to medium length of between 10 – 20 minutes as patients may not want to be held up in the clinic for a long time and the clinic staff may be overwhelmed with routine work. If the instrument is in a local language, it can be handed to patients to fill in as they are waiting to be seen by clinic staff. The clinic staff can then check it for completion and only help those patients who are unable to fill it themselves. An instrument with these features would help the TB program understand the subjective view of the patients' quality of life during combined TB and HIV treatment.

We also concluded that the use of urine LAM for diagnosis of smear-negative TB in HIV-infected patients is useful and may reduce the overuse of antibiotics.

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Appendices

Appendix A

Genotype table

GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
									ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20000	154	TT	TT	CC	AG	AA	GG	220100	282/341/590	282/341	5J/5K	282/341/590	282/341	5J/5K	1.000
GS20001	213	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.999
GS20002	214	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.999
GS20003	128	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20004	245	CC	TT	CC	AG	AA	GG	020100	341/590	341	5E/5D	341/590	341	5E/5D	1.000
GS20005	200	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.999
GS20006	199	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20007	182	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20008	110	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.999
GS20009	257	CC	TT	CC	GG	GG	GG	020020	341/803	341/803	5C/5C	341/803	341/803	5C/5C	1.000
GS20010	274	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.830
GS20011	215	CT	CT	CT	GG	AG	GG	111010	282/341/481/803		5G/4	282/341	481/803	5K/12C	0.883
GS20012	137	TT	TT	CC	AG	AA	GG	220100	282/341/590	282/341	5J/5K	282/341/590	282/341	5J/5K	1.000
GS20013	157	CC	TT	CC	AG	AG	GG	020110	341/803	341/590	5C/5E	341/803	341/590	5E/5C	0.938
GS20014	162	CT	CT	CT	GG	GG	GG	111020	282/341/481/803	803	5G/12A	282/341/803	481/803	5T/12C	0.607
GS20015	210	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20016	204	TT	TT	CC	AG	AA	GG	220100	282/341/590	282/341	5J/5K	282/341/590	282/341	5J/5K	1.000
GS20017	250	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.975
GS20018	205	CT	TT	CC	GG	AG	GG	120010	282/341/803	341	5J/5D	282/341	341/803	5K/5C	0.856
GS20019	127	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998

									HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20020	218	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20021	109	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.956
GS20022	144	CC	CC	TT	GG	GG	GG	002020	481/803	481/803	12C/12C	481/803	481/803	12C/12C	1.000
GS20023	211	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20024	167	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20025	153	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20026	219	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341	803	5D/12A	0.874
GS20027	134	TT	TT	CC	AG	AA	GG	220100	282/341/590	282/341	5J/5K	282/341/590	282/341	5J/5K	1.000
GS20028	241	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.830
GS20029	201	CT	CT	CC	GG	AG	GG	110010	282/341/803		5T/4	282/341	803	5K/12A	0.962
GS20030	148	CT	CT	CT	GG	AG	GG	111010	282/341/481/803		5G/4	282/341	481/803	5K/12C	0.881
GS20031	168	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20032	103	CT	CT	CT	GG	AG	GG	111010	282/341/481/803		5G/4	282/341	481/803	5K/12C	0.882
GS20033	188	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.830
GS20034	122	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.824
GS20035	242	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998
GS20036	170	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20037	141	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.975
GS20038	171	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.830
GS20039	230	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.831

									HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20041	32006	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.975
GS20042	32062	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20043	32112	CT	TT	CC	GG	AG	GG	120010	282/341/803	341	5K/5D	282/341	341/803	5K/5C	0.857
GS20044	32103	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20045	32117	CC	CT	CC	GG	GG	GG	010020	341/803	803	5C/12A	341/803	803	5C/12A	1.000
GS20046	32047	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998
GS20047	32072	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20048	32078	CC	CC	TT	GG	GG	GG	002020	481/803	481/803	12C/12C	481/803	481/803	12C/12C	1.000
GS20049	32086	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20050	32004	CC	CT	CT	GG	AG	GG	011010	341/481/803		5H/4	341	481/803	5D/12C	0.983
GS20053	32045	CT	TT	CC	AA	AA	GG	120200	282/341/590	341/590	5J/5E	282/341/590	341/590	5J/5E	1.000
GS20054	32005	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20055	32064	CC	TT	CC	AG	AA	GG	021100	341/590	341	5E/5D	341/590	341	5E/5D	1.000
GS20056	32069	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20057	32053	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20058	32010	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20059	32038	CC	CT	CC	GG	AG	GG	010010	341/803		5C/4	341	803	5D/12A	0.874
GS20060	32033	CC	CT	CT	AG	AG	GG	011110	341/803	481/590	5C/6E	341/590	481/803	5E/12C	0.973
GS20061	32074	CC	CT	CT	GG	GG	GG	010020	341/803	803	5C/12A	341/803	481/803	5C/12C	0.998
GS20062	32059	CC	CT	CT	AG	AG	GG	011110	481/803	341/590	12C/5E	341/590	481/803	5E/12C	0.976
GS20063	32082	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998

									HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20064	32100	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20065	32013	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20066	32020	TT	TT	CC	AG	AG	GG	220110	282/341/590	282/341/803	5J/5T	282/341/590	282/341/803	5J/5T	0.942
GS20067	32009	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998
GS20068	32096	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20069	32025	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5B/12A	341/803	481/803	5C/12C	0.998
GS20070	32031	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20071	32089	CT	TT	CC	GG	AG	GG	120010	282/341/803	341	5T/5D	282/341	341/803	5K/5C	0.874
GS20072	32012	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20073	32050	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20074	32042	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.975
GS20075	32054	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000
GS20076	32057	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5B/12A	341/803	481/803	5C/12C	0.998
GS20077	32061	CC	CT	CT	GG	AG	GG	011010	341/481/803		5H/4	341	481/803	5D/12C	0.893
GS20078	32060	CC	CT	CC	GG	GG	GG	010020	341/803	803	5C/12A	341/803	803	5C/12A	1.000
GS20079	32065	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20080	32101	CC	TT	CC	AG	AA	GG	020100	341	341/590	5D/5E	341/590	341	5E/5D	1.000
GS20081	32084	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20082	32093	CT	TT	CC	AG	AA	GG	120100	282/341/590	341	5J/5D	282/341/590	341	5J/5D	0.829
GS20083	32108	CT	TT	CC	GG	AG	GG	120010	282/341/803	341	5T/5D	282/341	341/803	5K/5C	0.853
GS20084	32008	BLANK	TT	CC	GG	AA	GG	-20000	341	341	5D/5D	341	341	5K/5D	0.476

									HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20085	32113	CC	TT	CC	GG	GG	GG	020020	341/803	341/803	5C/5C	341/803	341/803	5C/5C	1.000
GS20086	32019	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.975
GS20087	32024	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20088	32026	CT	TT	CC	GG	GG	GG	120020	282/341/803	341/803	5T/5C	282/341/803	341/803	5T/5C	1.000
GS20089	32011	CC	CT	CT	AG	AG	GG	011110	341/481/803	590	5H/6B	341/590	481/803	5E/12C	0.977
GS20090	32032	CC	TT	CC	GG	BLANK	GG	0200-0	341	341	5D/5D	341	341/803	5D/5C	0.486
GS20091	32036	CC	CT	CT	GG	AG	GG	011010	341/481/803		5H/4	341	481/803	5D/12C	0.894
GS20092	32017	CC	CC	CT	GG	GG	GG	001020	481/803	803	12C/12A	803	481/803	12A/12C	1.000
GS20093	32018	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20094	32039	TT	TT	CC	AA	AA	GG	220200	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	1.000
GS20095	32022	CC	CC	TT	GG	GG	GG	002020	481/803	481/803	12C/12C	481/803	481/803	12C/12C	1.000
GS20096	32040	CT	CT	CT	AG	AG	GG	111110	282/341/590	481/803	5J/12C	282/341/590	481/803	5J/12C	0.976
GS20097	32016	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20098	32116	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998
GS20099	32043	CT	TT	CC	GG	AG	AG	120011	282/341/803	341/857	5KA/5S	282/341/857	341/803	5KA/5C	0.972
GS20100	32044	CC	CC	CT	GG	AA	GG	001000	481		11A/4		481	4/11A	1.000
GS20101	32049	CC	CT	CC	AG	AG	GG	010110	341/803	590	5C/6B	341/590	803	5E/12A	0.939
GS20102	32027	CT	TT	CC	GG	AG	GG	120010	282/341/803	341	5J/5D	282/341	341/803	5K/5C	0.855
GS20103	32067	CC	CC	TT	GG	GG	GG	002020	481/803	481/803	12C/12C	481/803	481/803	12C/12C	1.000
GS20104	32070	TT	TT	CC	AA	BLANK	GG	2202-0	282/341/590	282/341/590	5J/5J	282/341/590	282/341/590	5J/5J	0.975
GS20105	32073	CT	TT	CC	GG	AA	GG	120000	282/341	341	5K/5D	282/341	341	5K/5D	1.000

									HAPLOTYPE BY HUMAN NAT2 ALLELES 2016			HAPLOTYPE BY PHASE 2.1.1			
GS NUMBER	STUDY NUMBER	C282T	C341T	C481T	G590A	A803G	G857A	OBSERVED DIPLOTYPE	ALLELE 1	ALLELE 2		ALLELE 1	ALLELE 2	PHASE	P
GS20106	32075	CC	CC	CC	GG	GG	GG	000020	803	803	12A/12A	803	803	12A/12A	1.000
GS20107	32079	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20108	32083	CC	CC	TT	GG	AG	GG	002010	481/803	481	12C/11A	481	481/803	11A/12C	1.000
GS20109	32023	CT	CT	CC	AG	AG	GG	110110	282/341/590	803	5J/12A	282/341	481/803	5K/12C	0.881
GS20110	32098	CT	CT	CT	GG	AG	GG	111010	282/341/481/803		5G/4	341	341/803	5D/5C	1.000
GS20111	32088	CC	TT	CC	GG	AG	GG	020010	341/803	341	5C/5D	341	341/803	5D/5C	1.000
GS20112	32002	CT	CT	CT	GG	AG	GG	111010	282/341/481/803		5G/4	281/341	481/803	5K/12C	0.882
GS20113	32007	TT	CT	CT	GG	GG	GG	211020	282/341/481/803	282/803	5G/12B	282/341/803	282/481/803	5T/12M	0.999
GS20114	32015	CT	TT	CC	AG	AG	GG	120110	282/341/590	341/803	5J/5C	282/341/590	341/803	5J/5C	0.955
GS20115	32021	CC	CT	CC	AG	AG	GG	010110	341/803	590	5C/6B	341/590	803	5E/12A	0.936
GS20116	32001	CC	TT	CC	GG	GG	GG	020020	341/803	341/803	5C/5C	341/803	341/803	5C/5C	1.000
GS20117	32029	CC	CT	BLANK	GG	GG	GG	01-020	341/803	803	5C/12A	341/803	481/803	5C/12C	0.754
GS20118	32058	TT	CT	CT	AG	AG	GG	211110	282/341/481/803	282/590	5G/6A	282/341/590	282/481/803	5J/12M	0.999
GS20119	32091	CC	CT	CT	GG	GG	GG	011020	341/481/803	803	5H/12A	341/803	481/803	5C/12C	0.998
GS20120	32003	TT	TT	CC	GG	AA	AG	220000	282/341/857	282/341	5KA/5K	282/341	282/341/857	5KA/5K	1.000
GS20121	32090	CC	TT	CC	GG	AA	GG	020000	341	341	5D/5D	341	341	5D/5D	1.000
GS20122	32095	CC	CC	TT	GG	AG	GG	002010	481/803	481	12C/11A	481	481/803	11A/12C	1.000

Appendix B

FAHI instrument

FUNCTIONAL ASSESSMENT OF HIV INFECTION (FAHI) AND BEHAVIORAL RISK ASSESSMENT QUESTIONNAIRE

Below is a list of statements that other people with your illness have said are important. **By circling one (1) number per line, please indicate how true each statement has been for you during the past 7 days.**

	PHYSICAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4
B1	*I have been short of breath	0	1	2	3	4
B8	I am bothered by a change in weight	0	1	2	3	4
BMT6	I get tired easily	0	1	2	3	4
HI7	I feel fatigued	0	1	2	3	4
HI12	I feel weak all over	0	1	2	3	4
L2	*I have been coughing	0	1	2	3	4

* Items are not scored in current FAHI version.

US English

3/20/03

By circling one (1) number per line, please indicate how true each statement has been for you during the past 7 days.

	EMOTIONAL WELL-BEING/ LIVING WITH HIV	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GE1	I feel sad	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4
HI1	I am unhappy with my appearance	0	1	2	3	4
HI2	It is hard to tell other people about my infection	0	1	2	3	4
HI4	I worry about spreading my infection	0	1	2	3	4
HI5	I am concerned about what the future holds for me	0	1	2	3	4
B7	I worry about the effect of stress on my illness	0	1	2	3	4
HI10	I am embarrassed by my illness	0	1	2	3	4

By circling one (1) number per line, please indicate how true each statement has been for you during the past 7 days.

	FUNCTIONAL AND GLOBAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4
B4	I feel sexually attractive	0	1	2	3	4
C6	I have a good appetite	0	1	2	3	4
HI6	I feel motivated to do things	0	1	2	3	4
HI11	I am hopeful about the future	0	1	2	3	4

By circling one (1) number per line, please indicate how true each statement has been for you during the past 7 days.

Additional questions

	Central nervous system	**Not at all**	**A little bit**	**Some -what**	**Quite a bit**	**Very much**
CNS1	I have been waking up during sleep	0	1	2	3	4
CNS2	I have had abnormal dreams	0	1	2	3	4
CNS3	I have felt sleepy during the day	0	1	2	3	4
CNS4	I drink alcohol	0	1	2	3	4
CNS5	I have problems with balancing	0	1	2	3	4
CNS6	I have had dizziness	0	1	2	3	4
CNS7	I have problems with my hearing	0	1	2	3	4
CNS8	I have pain in my feet	0	1	2	3	4
CNS9	My feet are numb	0	1	2	3	4
CNS10	I am worried that my body is changing shape	0	1	2	3	4